NATIONAL INSTITUTES OF HEALTH

GUIDELINES FOR

RESEARCH INVOLVING RECOMBINANT DNA MOLECULES

JUNE 1976

INDEX

I. Introduction

The purpose of these guidelines is to recommend safeguards for research on recombinant DNA molecules to the National Institutes of Health and to other institutions that support such research. In this context we define recombinant DNAs as molecules that consist of different segments of DNA which have been joined together in cell-free systems, and which have the capacity to infect and replicate in some host cell, either autonomously or as an integrated part of the host's genome.

This is the first attempt to provide a detailed set of guidelines for use by study sections as well as practicing scientists for evaluating research on recombinant DNA molecules. We cannot hope to anticipate all possible lines of imaginative research that are possible with this powerful new methodology. Nevertheless, a considerable volume of written and verbal contributions from scientists in a variety of disciplines has been received. In many instances the views presented to us were contradictory. At present, the hazards may be guessed at, speculated about, or voted upon, but they cannot be known absolutely in the absence of firm experimental data--and, unfortunately, the needed data were, more often than not, unavailable. Our problem then has been to construct guidelines that allow the promise of the methodology to be realized while advocating the considerable caution that is demanded by what we and others view as potential hazards.

In designing these guidelines we have adopted the following principles, which are consistent with the general conclusions that were formulated at the International Conference on Recombinant DNA Molecules held at Asilomar

Conference Center, Pacific Grove, California, in February 1975 (3): (i) There are certain experiments for which the assessed potential hazard is so serious that they are not to be attempted at the present time. (ii) The remainder can be undertaken at the present time provided that the experiment is justifiable on the basis that new knowledge or benefits to humankind will accrue that cannot readily be obtained by use of conventional methodology and that appropriate safeguards are incorporated into the design and execution of the experiment. In addition to an insistence on the practice of good microbiological techniques, these safeguards consist of providing both physical and biological barriers to the dissemination of the potentially hazardous agents. (iii) The level of containment provided by these barriers is to match the estimated potential hazard for each of the different classes of recombinants. For projects in a given class, this level is to be highest at initiation and modified subsequently only if there is a substantiated change in the assessed risk or in the applied methodology. (iv) The guidelines will be subjected to periodic review (at least annually) and modified to reflect improvements in our knowledge of the potential biohazards and of the available safeguards.

In constructing these guidelines it has been necessary to define boundary conditions for the different levels of physical and biological containment and for the classes of experiments to which they apply. We recognize that these definitions do not take into account existing and anticipated special procedures and information that will allow particular

experiments to be carried out under different conditions than indicated here without sacrifice of safety. Indeed, we urge that individual investigators devise simple and more effective containment procedures and that study sections give consideration to such procedures which may allow change in the containment levels recommended here.

It is recommended that all publications dealing with recombinant DNA work include a description of the physical and biological containment procedures practiced, to aid and forewarn others who might consider repeating the work.

II. Containment

Effective biological safety programs have been operative in a variety of laboratories for many years. Considerable information therefore already exists for the design of physical containment facilities and the selection of laboratory procedures applicable to organisms carrying recombinant DNAs (4-17). The existing programs rely upon mechanisms that, for convenience, can be divided into two categories: (i) a set of standard practices that are generally used in microbiological laboratories, and (ii) special procedures, equipment, and laboratory installations that provide physical barriers which are applied in varying degrees according to the estimated biohazard.

Experiments on recombinant DNAs by their very nature lend themselves to a third containment mechanism--namely, the application of highly specific biological barriers. In fact, natural barriers do exist which either limit the infectivity of a vector or vehicle (plasmid, bacteriophage or virus) to specific hosts, or its dissemination and survival in the environment. The vectors that provide the means for replication of the recombinant DNAs and/or the host cells in which they replicate can be genetically designed to decrease by many orders of magnitude the probability of dissemination of recombinant DNAs outside the laboratory.

As these three means of containment are complementary, different levels of containment appropriate for experiments with different recombinants can be established by applying different combinations of the physical and biological barriers to a constant use of the standard practices. We consider these categories of containment separately here in order that such combinations can be conveniently expressed in the guidelines for research on the different kinds of recombinant DNAs (Section III).

A. Standard practices and training - The first principle of containment is a strict adherence to good microbiological practices (4-13). Consequently, all personnel directly or indirectly involved in experiments on recombinant DNAs must receive adequate instruction. This should include at least training in aseptic techniques and instruction in the biology of the organisms used in the experiments so that the potential biohazards can be understood and appreciated.

Any research group working with agents with a known or potential biohazard should have an emergency plan which describes the procedures to be followed if an accident contaminates personnel or environment. The principal investigator must ensure that everyone in the laboratory is familiar with both the potential hazards of the work and the emergency plan. If a research group is working with a known pathogen for which an effective vaccine is available, all workers should be immunized. Serological monitoring, where appropriate, should be provided.

B. Physical containment levels - A variety of combinations (levels) of special practices, equipment, and laboratory installations that provide additional physical barriers can be formed. For example, 31 combinations are listed in "Laboratory Safety at the Center for Disease Control" (4); four levels are associated with the "Classification of Etiologic Agents on the Basis of Hazard" (5), four levels were recommended in the "Summary Statement of the Asilomar Conference on Recombinant DNA Molecules" (3); and the National Cancer Institute uses three levels for research on oncogenic viruses (6). We emphasize that tnese are an aid to, and not a substitute for, good technique. Personnel must be competent in the effective use of all

equipment needed for the required containment level as described below. We define only four levels of physical containment here, both because the accuracy with which one can presently assess the biohazards that may result from recombinant DNAs does not warrant a more detailed classification, and because additional flexibility can be obtained by combination of the physical with the biological barriers. Though different in detail, these four levels (P1 < P2 < P3 < P4) approximate those given for human etiologic agents by the Center for Disease Control (i.e., classes 1 through 4; ref. 5), in the Asilomar summary statement (i.e., minimal, low, moderate, and high; ref. 3), and by the National Cancer Institute for oncogenic viruses (i.e., low, moderate, and high; ref. 6), as is indicated by the P-number or adjective in the following headings. It should be emphasized that the descriptions and assignments of physical containment detailed below are based on existing approaches to containment of hazardous organisms.

We anticipate, and indeed already know of, procedures (14) which enhance physical containment capability in novel ways. For example, miniaturization of screening, handling, and analytical procedures provides substantial containment of a given host-vector system. Thus, such procedures should reduce the need for the standard types of physical containment, and such innovations will be considered by the Recombinant DNA Molecule Program Advisory Committee.

The special practices, equipment and facility installations indicated for each level of physical containment are required for the safety of laboratory workers, other persons, and for the protection of the environment. Optional items have been excluded; only those items deemed absolutely necessary for safety are presented. Thus, the listed requirements present basic safety

criteria for each level of physical containment. Other microbiological practices and laboratory techniques which promote safety are to be encouraged. Additional information giving further guidance on physical containment is provided in a supplement to the guidelines (Appendix D).

P1 Level (Minimal) - A laboratory suitable for experiments involving recombinant DNA molecules requiring physical containment at the P1 level is a laboratory that possesses no special engineering design features. It is a laboratory commonly used for microorganisms of no or minimal biohazard under ordinary conditions of handling. Work in this laboratory is generally conducted on open bench tops. Special containment equipment is neither required nor generally available in this laboratory. The laboratory is not separated from the general traffic patterns of the building. Public access is permitted.

The control of biohazards at the P1 level is provided by standard microbiological practices of which the following are examples: (i) Laboratory doors should be kept closed while experiments are in progress. (ii) Work surfaces should be decontaminated daily and following spills of recombinant DNA materials. (iii) Liquid wastes containing recombinant DNA materials should be decontaminated before disposal. (iv) Solid wastes contaminated with recombinant DNA materials should be decontaminated or packaged in a durable leak-proof container before removal from the laboratory. (v) Although pipetting by mouth is permitted, it is preferable that mechanical pipetting devices be used. When pipetting by mouth, cotton-plugged pipettes shall be employed. (vi) Eating, drinking, smoking, and storage of food in the working area should be discouraged. (vii) Facilities to wash hands should

be available. (viii) An insect and rodent control program should be provided. (ix) The use of laboratory gowns, coats, or uniforms is discretionary with the laboratory supervisor.

P2 Level (Low) - A laboratory suitable for experiments involving recombinant DNA molecules requiring physical containment at the P2 level is similar in construction and design to the P1 laboratory. The P2 laboratory must have access to an autoclave within the building; it may have a Biological Safety Cabinet.[1] Work which does not produce a considerable aerosol is conducted on the open bench. Although this laboratory is not separated from the general traffic patterns of the building, access to the laboratory is limited when experiments requiring P2 level physical containment are being conducted. Experiments of lesser biohazard potential can be carried out concurrently in carefully demarcated areas of the same laboratory.

The P2 laboratory is commonly used for experiments involving microorganisms of low biohazard such as those which have been classified by the Center for Disease Control as Class 2 agents (5).

The following practices shall apply to all experiments requiring P2 level physical containment: (i) Laboratory doors shall be kept closed while experiments are in progress. (ii) Only persons who have been advised of the potential biohazard shall enter the laboratory. (iii) Children under 12 years of age shall not enter the laboratory. (iv) Work surfaces shall be decontaminated daily and immediately following spills of recombinant DNA materials. (v) Liquid wastes of recombinant DNA materials shall be decontaminated before disposal. (vi) Solid wastes contaminated with recombinant DNA materials shall be decontaminated or packaged in a durable leak-proof

container before removal from the laboratory. Packaged materials shall be disposed of by incineration or sterilized before disposal by other methods. Contaminated materials that are to be processed and reused (i.e., glassware) shall be decontaminated before removal from the laboratory. (vii) Pipetting by mouth is prohibited; mechanical pipetting devices shall be used. (viii) Eating, drinking, smoking, and storage of food are not permitted in the working area. (ix) Facilities to wash hands shall be available within the laboratory. Persons handling recombinant DNA materials should be encouraged to wash their hands frequently and when they leave the laboratory. (x) An insect and rodent control program shall be provided. (xi) The use of laboratory gowns, coats, or uniforms is required. Such clothing shall not be worn to the lunch room or outside the building. (xii) Animals not related to the experiment shall not be permitted in the laboratory. (xiii) Biological Safety Cabinets[1] and/or other physical containment equipment shall be used to minimize the hazard of aerosolization of recombinant DNA materials from operations or devices that produce a considerable aerosol (e.g., blender, lyophilizer, sonicator, shaking machine, etc.). (xiv) Use of the hypodermic needle and syringe shall be avoided when alternate methods are available.

P3 Level (Moderate) - A laboratory suitable for experiments involving recombinant DNA molecules requiring physical containment at the P3 level has special engineering design features and physical containment equipment. The laboratory is separated from areas which are open to the general public. Separation is generally achieved by controlled access corridors, air locks, locker rooms or other double-doored facilities which are not available for use by the general public. Access to the laboratory is controlled. Biological

Safety Cabinets[1] are available within the controlled laboratory area. An autoclave shall be available within the building and preferably within the controlled laboratory area. The surfaces of walls, floors, bench tops, and ceilings are easily cleanable to facilitate housekeeping and space decontamination.

Directional air flow is provided within the controlled laboratory area. The ventilation system is balanced to provide for an inflow of supply air from the access corridor into the laboratory. The general exhaust air from the laboratory is discharged outdoors and so dispersed to the atmosphere as to prevent reentry into the building. No recirculation of the exhaust air shall be permitted without appropriate treatment.

No work in open vessels involving hosts or vectors containing recombinant DNA molecules requiring P3 physical containment is conducted on the open bench. All such procedures are confined to Biological Safety Cabinets.[1]

The following practices shall apply to all experiments requiring P3 level physical containment: (i) The universal biohazard sign is required on all laboratory access doors. Only persons whose entry into the laboratory is required on the basis of program or support needs shall be authorized to enter. Such persons shall be advised of the potential biohazards before entry and they shall comply with posted entry and exit procedures. Children under 12 years of age shall not enter the laboratory. (ii) Laboratory doors shall be kept closed while experiments are in progress. (iii) Biological Safety Cabinets[1] and other physical containment equipment shall be used for all procedures that produce aerosols of recombinant DNA materials (e.g., pipetting,

plating, flaming, transfer operations, grinding, blending, drying, sonicating, shaking, etc.). (iv) The work surfaces of Biological Safety Cabinets[1] and other equipment shall be decontaminated following the completion of the experimental activity contained within them. (v) Liquid wastes containing recombinant DNA materials shall be decontaminated before disposal. Solid wastes contaminated with recombinant DNA materials shall be decontaminated or packaged in a durable leak-proof container before removal from the laboratory. Packaged material shall be sterilized before disposal. Contaminated materials that are to be processed and reused (i.e., glassware) shall be sterilized in the controlled laboratory area or placed in a durable leak-proof container before removal from the controlled laboratory area. This container shall be sterilized before the materials are processed. (vii) Pipetting by mouth is prohibited; mechanical pipetting devices shall be used. (viii) Eating, drinking, smoking, and storage of food are not permitted in the laboratory. (ix) Facilities to wash hands shall be available within the laboratory. Persons shall wash hands after experiments involving recombinant DNA materials and before leaving the laboratory. (x) An insect and rodent control program shall be provided. (xi) Laboratory clothing that protects street clothing (i.e., long sleeve solid-front or wrap-around gowns, no-button or slipover jackets, etc.) shall be worn in the laboratory. FRONT-BUTTON LABORATORY COATS ARE UNSUITABLE. Gloves shall be worn when handling recombinant DNA materials. Provision for laboratory shoes is recommended. Laboratory clothing shall not be worn outside the laboratory and shall be decontaminated before it is sent to the laundry. (xii) Raincoats, overcoats, topcoats, coats, hats, caps, and such street outerwear shall not be kept in the laboratory.

(xiii) Animals and plants not related to the experiment shall not be permitted in the laboratory. (xiv) Vacuum lines shall be protected by filters and liquid traps. (xv) Use of the hypodermic needle and syringe shall be avoided when alternate methods are available. (xvi) If experiments of lesser biohazard potential are to be conducted in the same laboratory concurrently with experiments requiring P3 level physical containment they shall be conducted only in accordance with all P3 level requirements. (xvii) Experiments requiring P3 level physical containment can be conducted in laboratories where the directional air flow and general exhaust air conditions described above cannot be achieved, provided that this work is conducted in accordance with all other requirements listed and is contained in a Biological Safety Cabinet[1] with attached glove ports and gloves. All materials before removal from the Biological Safety Cabinet[1] shall be sterilized or transferred to a non-breakable, sealed container, which is then removed from the cabinet through a chemical decontamination tank, autoclave, ultraviolet air lock, or after the entire cabinet has been decontaminated.

P4 Level (High) - Experiments involving recombinant DNA molecules requiring physical containment at the P4 level shall be confined to work areas in a facility of the type designed to contain microorganisms that are extremely hazardous to man or may cause serious epidemic disease. The facility is either a separate building or it is a controlled area, within a building, which is completely isolated from all other areas of the building. Access to the facility is under strict control. A specific facility operations manual is available. Class III Biological Safety Cabinets[1] are available within work areas of the facility.

A P4 facility has engineering features which are designed to prevent the escape of microorganisms to the environment (14, 15, 16, 17). These features

include: (i) monolithic walls, floors, and ceilings in which all penetrations such as for air ducts, electrical conduits, and utility pipes are sealed to assure the physical isolation of the work area and to facilitate housekeeping and space decontamination; (ii) air locks through which supplies and materials can be brought safely into the facility; (iii) contiguous clothing change and shower rooms through which personnel enter into and exit from the facility; (iv) double-door autoclaves to sterilize and safely remove wastes and other materials from the facility; (v) a biowaste treatment system to sterilize liquid effluents if facility drains are installed; (vi) a separate ventilation system which maintains negative air pressures and directional air flow within the facility; and (vii) a treatment system to decontaminate exhaust air before it is dispersed to the atmosphere. A central vacuum utility system is not encouraged; if one is installed, each branch line leading to a laboratory shall be protected by a high efficiency particulate air filter.

The following practices shall apply to all experiments requiring P4 level physical containment: (i) The universal biohazard sign is required on all facility access doors and all interior doors to individual laboratory rooms where experiments are conducted. Only persons whose entry into the facility or individual laboratory rooms is required on the basis of program or support needs shall be authorized to enter. Such persons shall be advised of the potential biohazards and instructed as to the appropriate safeguards to ensure their safety before entry. Such persons shall comply with the instructions and all other posted entry and exit procedures. Under no condition shall children under 15 years of age be allowed entry. (ii) Personnel shall enter into and exit from the facility only through the clothing change and shower

rooms. Personnel shall shower at each exit from the facility. The air locks shall not be used for personnel entry or exit except for emergencies. (iii) Street clothing shall be removed in the outer facility side of the clothing change area and kept there. Complete laboratory clothing including undergarments, pants and shirts or jumpsuits, shoes, head cover, and gloves shall be provided and used by all persons who enter into the facility. Upon exit, this clothing shall be stored in lockers provided for this purpose or discarded into collection hampers before personnel enter into the shower area. (iv) Supplies and materials to be taken into the facility shall be placed in an entry air lock. After the outer door (opening to the corridor outside of facility) has been secured, personnel occupying the facility shall retrieve the supplies and materials by opening the interior air lock door. This door shall be secured after supplies and materials are brought into the facility. (v) Doors to laboratory rooms within the facility shall be kept closed while experiments are in progress. (vi) Experimental procedures requiring P4 level physical containment shall be confined to Class III Biological Safety Cabinets.[1] All materials, before removal from these cabinets, shall be sterilized or transferred to a non-breakable sealed container, which is then removed from the system through a chemical decontaminated tank, autoclave, or after the entire system has been decontaminated. (vii) No materials shall be removed from the facility unless they have been sterilized or decontaminated in a manner to prevent the release of agents requiring P4 physical containment. All wastes and other materials and equipment not damaged by high temperature or steam shall be sterilized in the double-door autoclave. Biological materials to be removed from the facility shall be transferred to a non-breakable sealed

container which is then removed from the facility through a chemical decontamination tank or a chamber designed for gas sterilization. Other materials which may be damaged by temperature or steam shall be sterilized by gaseous or vapor methods in an air lock or chamber designed for this purpose. (viii) Eating, drinking, smoking, and storage of food are not permitted in the facility. Foot-operated water fountains located in the facility corridors are permitted. Separate potable water piping shall be provided for these water fountains. (ix) Facilities to wash hands shall be available within the facility. Persons shall wash hands after experiments. (x) An insect and rodent control program shall be provided. (xi) Animals and plants not related to the experiment shall not be permitted in the facility. (xii) If a central vacuum system is provided, each vacuum outlet shall be protected by a filter and liquid trap in addition to the branch line HEPA filter mentioned above. (xiii) Use of the hypodermic needle and syringe shall be avoided when alternate methods are available. (xiv) If experiments of lesser biohazard potential are to be conducted in the facility concurrently with experiments requiring P4 level containment, they shall be confined in Class I or Class II Biological Safety Cabinets[1] or isolated by other physical containment equipment. Work surfaces of Biological Safety Cabinets[1] and other equipment shall be decontaminated following the completion of the experimental activity contained within them. Mechanical pipetting devices shall be used. All other practices listed above with the exception of (vi) shall apply.

C. Shipment - To protect product, personnel, and the environment, all recombinant DNA material will be shipped in containers that meet the

requirements issued by the U.S. Public Health Service (Section 72.25 of Part 72, Title 42, Code of Federal Regulations), Department of Transportation (Section 173.387 (b) of Part 173, Title 49, Code of Federal Regulations) and the Civil Aeronautics Board (C.A.B. No. 82, Official Air Transport Restricted Articles Tariff No. 6-D) for shipment of etiologic agents. Labeling requirements specified in these Federal regulations and tariffs will apply to all viable recombinant DNA materials in which any portion of the material is derived from an etiologic agent listed in paragraph (c) of 42 CFR 72.25. Additional information on packing and shipping is given in a supplement to the guidelines (Appendix D, part X).

D. Biological containment levels - Biological barriers are specific to each host-vector system. Hence the criteria for this mechanism of containment cannot be generalized to the same extent as for physical containment. This is particularly true at the present time when our experience with existing host-vector systems and our predictive knowledge about projected systems are sparse. The classification of experiments with recombinant DNAs that is necessary for the construction of the experimental guidelines (Section III) can be accomplished with least confusion if we use the host-vector system as the primary element and the source of the inserted DNA as the secondary element in the classification. It is therefore convenient to specify the nature of the biological containment under host-vector headings such as those given below for Escherichia coli K-12.

III. Experimental Guidelines

A general rule that, though obvious, deserves statement is that the level of containment required for any experiment on DNA recombinants shall never be less than that required for the most hazardous component used to construct and clone the recombinant DNA (i.e., vector, host, and inserted DNA). In most cases the level of containment will be greater, particularly when the recombinant DNA is formed from species that ordinarily do not exchange genetic information. Handling the purified DNA will generally require less stringent precautions than will propagating the DNA. However, the DNA itself should be handled at least as carefully as one would handle the most dangerous of the DNAs used to make it.

The above rule by itself effectively precludes certain experiments--namely, those in which one of the components is in Class 5 of the "Classification of Etiologic Agents on the Basis of Hazard" (5), as these are excluded from the United States by law and USDA administrative policy. There are additional experiments which may engender such serious biohazards that they are not to be performed at this time. These are considered prior to presentation of the containment guidelines for permissible experiments.

A. Experiments that are not to be performed - We recognize that it can be argued that certain of the recombinants placed in this category could be adequately contained at this time. Nonetheless, our estimates of the possible dangers that may ensue if that containment fails are of

such a magnitude that we consider it the wisest policy to at least defer experiments on these recombinant DNAs until there is more information to accurately assess that danger and to allow the construction of more effective biological barriers. In this respect, these guidelines are more stringent than those initially recommended (1).

The following experiments are not to be initiated at the present time: (i) Cloning of recombinant DNAs derived from the pathogenic organisms in Classes 3, 4, and 5 of "Classification of Etiologic Agents on the Basis of Hazard" (5), or oncogenic viruses classified by NCI as moderate risk (6), or cells known to be infected with such agents, regardless of the host-vector system used. (ii) Deliberate formation of recombinant DNAs containing genes for the biosynthesis of potent toxins (e.g., botulinum or diphtheria toxins; venoms from insects, snakes, etc.). (iii) Deliberate creation from plant pathogens of recombinant DNAs that are likely to increase virulence and host range. (iv) Deliberate release into the environment of any organism containing a recombinant DNA molecule. (v) Transfer of a drug resistance trait to microorganisms that are not known to acquire it naturally if such acquisition could compromise the use of a drug to control disease agents in human or veterinary medicine or agriculture.

In addition, at this time large-scale experiments (e.g., more than 10 liters of culture) with recombinant DNAs known to make harmful products are not to be carried out. We differentiate between small- and large-scale experiments

with such DNAs because the probability of escape from containment barriers normally increases with increasing scale. However, specific experiments in this category that are of direct societal benefit may be excepted from this rule if special biological containment precautions and equipment designed for large-scale operations are used, and provided that these experiments are expressly approved by the Recombinant DNA Molecule Program Advisory Committee of NIH.

B. Containment guidelines for permissible experiments - It is anticipated that most recombinant DNA experiments initiated before these guidelines are next reviewed (i.e., within the year) will employ E. coli K-12 host-vector systems. These are also the systems for which we have the most experience and knowledge regarding the effectiveness of the containment provided by existing hosts and vectors necessary for the construction of more effective biological barriers.

For these reasons, E. coli K-12 appears to be the system of choice at this time, although we have carefully considered arguments that many of the potential dangers are compounded by using an organism as intimately connected with a man as is E. coli. Thus, while proceeding cautiously with E. coli, serious efforts should be made toward developing alternate host-vector systems; this subject is discussed in considerable detail in Appendix A.

We therefore consider DNA recombinants in E. coli K-12 before proceeding to other host-vector systems.

1. Biological containment criteria using E. coli K-12 host-vectors

EK1 host-vectors - These are host-vector systems that can be estimated to already provide a moderate level of containment, and include most of the presently available systems. The host is always E. coli K-12, and the vectors include nonconjugative plasmids [e.g., pSC101, ColE1 or derivatives thereof (19-26)] and variants of bacteriophage λ (27-29).

The E. coli K-12 nonconjugative plasmid system is taken as an example to illustrate the approximate level of containment referred to here. The available data from experiments involving the feeding of bacteria to humans and calves (30-32) indicate that E. coli K-12 did not usually colonize the normal bowel, and exhibited little, if any, multiplication while passing through the alimentary tract even after feeding high doses (i.e., 10^9 to 10^{10} bacteria per human or calf). However, general extrapolation of these results may not be warranted because the implantation of bacteria into the intestinal tract depends on a number of parameters, such as the nature of the intestinal flora present in a given invidivual and the physiological state of the inoculum. Moreover, since viable E. coli K-12 can be found in the feces after humans are fed 10^7 bacteria in broth (30) or 3×10^4 bacteria protected by suspension in milk (31), transductional and conjugational transfer of the plasmid vectors from E. coli K-12 to resident bacteria in the fecal matter before and after excretion must also be considered.

The nonconjugative plasmid vectors cannot promote their own transfer, but require the presence of a conjugative plasmid for mobilization and transfer to other bacteria. When present in the same cell with derepressed conjugative plasmids such as F or R1drd19, the nonconjugative ColE1, ColE1-trp and pSC101 plasmids are transferred to suitable recipient strains under ideal laboratory conditions at frequencies of about 0.5, 10^{-4} to 10^{-5}, and 10^{-6} per donor cell, respectively. These frequencies are reduced by another factor of 10^{2} to 10^{4} if the conjugative plasmid employed is repressed with respect to expression of donor fertility.

The experimental transfer system which most closely resembles nonconjugative plasmid transfer in nature is a triparental mating. In such matings, the bacterial cell possessing the nonconjugative plasmid must first acquire a conjugative plasmid from another cell before it can transfer the nonconjugative plasmid to a secondary recipient. With ColE1, the frequencies of transfer are 10^{-2} and 10^{-4} to 10^{-5} when using conjugative plasmid donors possessing derepressed and repressed plasmids, respectively. Mobilization of ColE1-trp and pSC101 under similar laboratory conditions is so low as to be usually undetectable (33). Since most conjugative plasmids in nature are repressed for expression of donor fertility, the frequency at which nonconjugative plasmids are mobilized and transferred by this sequence of events in vivo

is difficult to estimate. However, in calves fed on an antibiotic-supplemented diet, it has been estimated that such triparental nonconjugative R plasmid transfer occurs at frequencies of no more than 10^{-10} to 10^{-12} per 24 hours per calf (32). In terms of considering other means for plasmid transmission in nature, it should be noted that transduction does operate in vivo for Staphylococcus aureus (34) and probably for E. coli as well. However, no data are available to indicate the frequencies of plasmid transfer in vivo by either transduction or transformation.

These observations indicate the low probabilities for possible dissemination of such plasmid vectors by accidental ingestion, which would probably involve only a few hundred or thousand bacteria provided that at least the standard practices (Section II-A above) are followed, particularly the avoidance of mouth pipetting. The possibility of colonization and hence of transfer are increased, however, if the normal flora in the bowel is disrupted by, for example, antibiotic therapy (35). For this reason, persons receiving such therapy must not work with DNA recombinants formed with any E. coli K-12 host-vector system during the therapy period and for seven days thereafter; similarly, persons who have achlorhydria or who have had surgical removal of part of the stomach or bowel should avoid such work, as should those who require large doses of antacids.

The observations on the fate of E. coli K-12 in the human alimentary tract are also relevant to the containment of recombinant DNA formed with bacteriophage λ variants. Bacteriophage can escape from the laboratory either as mature infectious phage particles or in bacterial host cells in which the phage genome is carried as a plasmid or prophage. The fate of E. coli K-12 host cells carrying the phage genome as a plasmid or prophage is similar to that for plasmid-containing host cells as discussed above. The survival of the λ phage genome when released as infectious particles depends on their stability in nature, their infectivity and on the probability of subsequent encounters with naturally occurring λ-sensitive E. coli strains. Although the probability of survival of λ and its infection of resident intestinal E. coli in animals and humans has not been measured, it is estimated to be small given the high sensitivity of λ to the low pH of the stomach, the insusceptibility to λ infection of smooth E. coli cells (the type that normally resides in the gut), the infrequency of naturally occurring λ-sensitive E. coli (36) and the failure to detect infective λ particles in human feces after ingestion of up to 10^{11} λ particles (37). Moreover, λ particles are very sensitive to desiccation.

Establishment of λ as a stable lysogen is a frequent event (10^{0} to 10^{-1}) for the att^{+} int^{+} cI^{+} phage so that this mode of escape would be the preponderant laboratory hazard;

however, most EK1 λ vectors currently in use lack the att and int functions (27-29) thus reducing the probability of lysogenization to about 10^{-5} to 10^{-6} (38-40). The frequency for the conversion of λ to a plasmid state for persistence and replication is also only about 10^{-6} (41). Moreover, the routine treatment of phage lysates with chloroform (42) should eliminate all surviving bacteria including lysogens and λ plasmid carriers. Lysogenization could also occur when an infectious λ containing cloned DNA infects a λ-sensitive cell in nature, and recombines with a resident lambdoid prophage. Although λ-sensitive E. coli strains seem to be rare, a significant fraction do carry lambdoid prophages (43-44) and thus this route of escape should be considered.

While not exact, the estimates for containment afforded by using these host-vectors are at least as accurate as those for physical containment, and are sufficient to indicate that currently employed plasmid and λ vector systems provide a moderate level of biological containment. Other nonconjugative plasmids and bacteriophages that, in association with E. coli K-12 can be estimated to provide the same approximate level of moderate containment are included in the EK1 class.

EK2 host-vectors - These are host-vector systems that have been genetically constructed and shown to provide a high level of biological containment as demonstrated by data from suitable tests performed in the laboratory. The genetic modifications of the E. coli K-12 host and/or the plasmid or phage vector should not permit survival of a genetic marker carried on the vector, preferably a marker within an inserted DNA fragment, in other than specially designed and carefully regulated laboratory environments at a frequency greater than 10^{-8}. This measure of biological containment has been selected because it is a measurable entity. Indeed, by testing the contributions of preexisting and newly introduced genetic properties of vectors and hosts, individually or in various combinations, it should be possible to estimate with considerable precision, that the specially designed host-vector system can provide a margin of biological containment in excess of that required. For the time being, no host-vector system will be considered to be a bona fide EK2 host-vector system until it is so certified by the NIH Recombinant DNA Molecule Program Advisory Committee.

For EK2 host-vector systems in which the vector is a plasmid, no more than one in 10^8 host cells should be able to perpetuate the vector and/or a cloned DNA fragment under non-permissive conditions designed to represent the natural environment either by survival of the original host or as a consequence of transmission of the vector and/or a cloned DNA fragment by transformation, transduction or conjugation to a host with properties common to those in the natural environment.

In terms of potential EK2 plasmid-host systems, the following types of genetic modifications should reduce survival of cloned DNA. The examples given are for illustrative purposes and should

not be construed to encompass all possibilities. The presence of the non-conjugative plasmids ColE1-trp and pS101 in an E. coli K-12 strain possessing a mutation eliminating host-controlled restriction and modification (hsdS) results in about 10^2-fold reduction in mobilization to restriction-proficient recipients. The combination of the dapD8, ΔbioH-asd, Δgal-chl^r and rfb mutations in E. coli K-12 results in no detectable survivors in feces of rats following feeding by stomach tube of 10^{10} cells in milk and similarly leads to complete lysis of cells suspended in broth medium lacking diaminopimelic acid. E. coli K-12 strains with ΔthyA and deoC(dra) mutations undergo thymineless death in growth medium lacking thymine and give a 10^5-fold reduced survival during passage through the rat intestine compared to wild-type thy$^+$ E. coli K-12. (However, the ΔthyA mutation alone or in combination with a deoB(drm) mutation only reduces in vivo survival by a factor of 10^2.) Other host mutations, as yet untested, that might further reduce survival of the plasmid-host system or reduce plasmid transmission are: the combination polA(TS) recA(TS) ΔthyA which might interfere with ColE1 replication and lead to DNA degradation at body temperatures; Con$^-$ mutations that reduce the ability of conjugative plasmids to enter the plasmid-host complex and thus should reduce mobilization of the cloned DNA to other strains; and mutations that confer resistance to known transducing phages.

Mutations can also be introduced into the plasmid to cause it to be dependent on a specific host, to make its replication thermosensitive and/or to endow it with a killer capability such that all cells (other than its host) into which it might be transferred will not survive.

In the construction of EK2 plasmid-host systems it is important to use the most stable mutations available, preferably deletions. Obviously, the presence of all mutations contributing to higher degrees of biological containment must be verified periodically by appropriate tests. In testing the level of biological containment afforded by a proposed EK2 plasmid-host system, it is important to design relevant tests to evaluate the survival of the vector and/or a cloned DNA fragment under conditions that are possible in nature and that are also most advantageous for its perpetuation. For example, one might conduct a triparental mating with a primary donor possessing a derepressed F-type or I-type conjugative plasmid, the safer host with Δ*bioH-asd*, *dapD8*, Δ*gal-chl*^{r}, *rfb*, Δ*thyA*, *deoC*, *trp* *and* *hsdS* *mutations and a plasmid vector carrying an easily detectable marker such as for ampicillin resistance or an inserted gene such as* *trp*$^{+}$, *and a secondary recipient that is Su*$^{+}$ *hsdS* *trp* *(i.e., permissive for the recombinant plasmid). Such matings would be conducted in a medium lacking diaminopimelic acid and thymine and survival of the Ap*^{r} *or* *trp*$^{+}$ *marker in any of the three strains followed as a function of time. Survival of the vector and/or a cloned marker by transduction could also be evaluated by introducing a known generalized transducing phage into*

the system. Similar experiments should also be done using a secondary recipient that is restrictive for the plasmid vector as well as with primary donors possessing repressed conjugative plasmids with incompatibility group properties like those commonly found in enteric microorganisms. Since a common route of escape of plasmid-host systems in the laboratory might be by accidental ingestion, it is suggested that the same types of experiments be conducted in suitable animal-model systems. In addition to these tests on survival of the vector and/or a cloned DNA fragment, it would be useful to determine the survival of the host strain under nongrowth conditions such as in water and as a function of drying time after a culture has been spilled on a lab bench.

For EK2 host-vector systems in which the vector is a phage, no more than one in 10^8 phage particles should be able to perpetuate itself and/or a cloned DNA fragment under non-permissive conditions designed to represent the natural environment either (a) as a prophage or plasmid in the laboratory host used for phage propagation or (b) by surviving in natural environments and transferring itself and/or a cloned DNA fragment to a host (or its resident lambdoid prophage) with properties common to those in the natural environment.

In terms of potential EK2 λ-host systems, the following types of genetic modification should reduce survival of cloned DNA. The examples given are for illustrative purposes

and should not be construed to encompass all possibilities. The probability of establishing λ lysogeny in the normal laboratory host should be reduced by removal of the phage att site, the Int function, the repressor gene(s) and adding virulence-enhancing mutations. The frequency of plasmid formation, although normally already less than 10^{-6}, could be further reduced by defects in the p_R-Q region, including mutations such as vir-s, cro(TS), c17, ri^c, O(TS), P(TS), and nin. Moreover, chloroform treatment used routinely following cell lysis would reduce the number of surviving cells, including possible lysogens or plasmid carriers, by more than 10^8. The host may also be modified by deletion of the host λatt site and inclusion of one or more of the mutations described above for plasmid-host systems to further reduce the chance of formation and survival of any lysogen or plasmid carrier cell.

The survival of escaping phage and the chance of encountering a sensitive host in nature are very low, as discussed for EK1 systems. The infectivity of the phage particles could be further reduced by introducing mutations (e.g., suppressed ambers) which would make the phage particles extremely unstable except under special laboratory conditions (e.g., high concentrations of salts or putrescine). Another means would be to make the phage itself a two-component system, by eliminating the tail genes and reproducing the

phage as heads packed with DNA; when necessary and under specially controlled conditions, these heads could be made infective by adding tail preparations. An additional safety factor in this regimen is the extreme instability of the heads, unless they are stored in 10mM putrescine, a condition easy to obtain in the laboratory but not in nature. The propagation of the escaping phage in nature could further be blocked by adding various conditional mutations which would permit growth only under special laboratory conditions or in a special permissive laboratory host with suppressor or gro-type (mop, dnaB, rpoB) mutations. An additional safety feature would be the use of an r^- m^- (hsdS) laboratory host, which produces phage with unmodified DNA which should be restricted in r^+ m^+ bacteria that are probably prevalent in nature. The likelihood of recombination between the λ vector and lambdoid prophages which are present in some E. coli strains might be reduced by elimination of the Red function and the presence of the recombination-reducing Gam function together with mutations contributing to the high lethality of the λ phage. However, these second-order precautions might not be relevant if the stability and infectivity of the escaping λ particles are reduced by special mutations or by propagating the highly unstable heads.

Despite multiple mutations in the phage vectors and laboratory hosts, the yield of phage particles under suitable laboratory conditions should be high (10^{10}-10^{11} particles/ml).

This permits phage propagation in relatively small volumes and constitutes an additional safety feature.

The phenotypes and genetic stabilities of the mutations and chromosome alterations included in these λ-host systems indicate that containment well in excess of the required 10^{-8} or lower survival frequency for the λ vector with or without a cloned DNA fragment should be attained. Obviously the presence of all mutations contributing to this high degree of biological containment must be verified periodically by appropriate tests. Laboratory tests should be performed with the bacterial host to measure all possible routes of escape such as the frequency of lysogen formation, the frequency of plasmid formation and the survival of the lysogen or carrier bacterium. Similarly, the potential for perpetuation of a cloned DNA fragment carried by infectious phage particles can be tested by challenging typical wild-type E. coli strains or a λ-sensitive nonpermissive laboratory K-12 strain, especially one lysogenic for a lambdoid phage.

In view of the fact that accurate assessment of the probabilities for escape of infectious λ grown on r^- m^- Su^+ hosts is dependent upon the frequencies of r^-, Su^+, and λ-sensitive strains in nature, investigators need to screen E. coli strains for these properties. These data will also be useful in predicting frequencies of successful escape of plasmid cloning vectors harbored in r^- m^- Su^+ strains.

When any investigator has obtained data on the level of containment provided by a proposed EK2 system, these should be reported as rapidly as possible to permit general awareness and evaluation of the safety features of the new system. Investigators are also encouraged to make such new safer cloning systems generally available to other scientists. NIH will take appropriate steps to aid in the distribution of these safer vectors and hosts.

EK3 host-vectors - These are EK2 systems for which the specified containment shown by laboratory tests has been independently confirmed by appropriate tests in animals, including humans or primates, and in other relevant environments in order to provide additional data to validate the levels of containment afforded by the EK2 host-vector systems. Evaluation of the effects of individual or combinations of mutations contributing to the biological containment should be performed as a means to confirm the degree of safety provided and to further advance the technology of developing even safer vectors and hosts. For the time being, no host-vector system will be considered to be a bona fide EK3 host-vector system, until it is so certified by the NIH Recombinant DNA Molecule Program Advisory Committee.

2. Classification of experiments using the E. coli K-12 containment systems

In the following classification of containment criteria for different kinds of recombinant DNAs, the stated levels of physical and biological containment are minimums. Higher levels of biological containment (EK3 > EK2 > EK1) are to be used if they are available and are equally appropriate for the purposes of the experiment.

<a> Shotgun Experiments

These experiments involve the production of recombinant DNAs between the vector and the total DNA or (preferably) any partially purified fraction thereof from the specified cellular source.

(i) Eukaryotic DNA recombinants

Primates - P3 physical containment + an EK3 host-vector, or P4 physical containment + an EK2 host-vector, except for DNA from uncontaminated embryonic tissue or primary tissue cultures therefrom, and germ-line cells for which P3 physical containment + an EK2 host-vector can be used. The basis for the lower estimated hazard in the case of DNA from the latter tissues (if freed of adult tissue) is their relative freedom from horizontally acquired adventitious viruses.

Other mammals - P3 physical containment + an EK2 host-vector.

Birds - P3 physical containment + an EK2 host-vector.

Cold-blooded vertebrates - P2 physical containment + an EK2 host-vector except for embryonic or germ-line DNA which require P2 physical containment + an EK1 host-vector. If the eukaryote is known to produce a potent toxin, the containment shall be increased to P3 + EK2.

Other cold-blooded animals and lower eukaryotes - This large class of eukaryotes is divided into the following two groups:

(1) Species that are known to produce a potent toxin or are known pathogens (i.e., an agent listed in Class 2 of ref. 5 or a plant pathogen) or are known to carry such pathogenic agents must use P3 physical containment + an EK2 host-vector. Any species that has a

demonstrated capacity for carrying particular pathogenic agents is included in this group unless it has been shown that those organisms used as the source of DNA do not contain these agents; in this case they may be placed in the second group.

(2) The remainder of the species in this class can use P2 + EK1. However, any insect in this group should have been grown under laboratory conditions for at least 10 generations prior to its use as a source of DNA.

Plants - P2 physical containment + an EK1 host-vector. If the plant carries a known pathogenic agent or makes a product known to be dangerous to any species, the containment must be raised to P3 physical containment + an EK2 host-vector.

(ii) Prokaryotic DNA recombinants

Prokaryotes that exchange genetic information with E. coli[2] -

The level of physical containment is directly determined by the rule of the most dangerous component (see introduction to Section III). Thus P1 conditions can be used for DNAs from those bacteria in Class 1 of ref. 5 ("Agents of no or minimal hazard....") which naturally exchange genes with E. coli; and P2 conditions should be used for such bacteria if they fall in Class 2 of ref. 5 ("Agents of ordinary potential hazard...."), or are plant pathogens or symbionts. EK1 host-vectors can be used for all experiments requiring only P1 physical containment; in fact, experiments in this category can be performed with E. coli K-12 vectors exhibiting a lesser containment (e.g., conjugative plasmids) than EK1 vectors. Experiments with DNA from species requiring P2 physical containment which are of low pathogenicity (for example, enteropathogenic Escherichia

coli, Salmonella typhimurium, and Klebsiella pneumoniae) can use EK1 host-vectors, but those of moderate pathogenicity (for example, Salmonella typhi, Shigella dysenteriae type I, and Vibrio cholerae) must use EK2 host-vectors.[3] A specific example of an experiment with a plant pathogen requiring P2 physical containment + an EK2 host-vector would be cloning the tumor gene of Agrobacterium tumefaciens.

Prokaryotes that do not exchange genetic information with E. coli - The minimum containment conditions for this class consist of P2 physical containment + an EK2 host-vector or P3 physical containment + an EK1 host-vector, and apply when the risk that the recombinant DNAs will increase the pathogenicity or ecological potential of the host is judged to be minimal. Experiments with DNAs from pathogenic species (Class 2 ref. 5 plus plant pathogens) must use P3 + EK2.

(iii) Characterized clones of DNA recombinants derived from shotgun experiments

When a cloned DNA recombinant has been rigorously characterized[4] and there is sufficient evidence that it is free of harmful genes,[4] then experiments involving this recombinant DNA can be carried out under P1 + EK1 conditions if the inserted DNA is from a species that exchanges genes with E. coli, and under P2 + EK1 conditions if not.

<b> Purified cellular DNAs other than plasmids, bacteriophages, and other viruses

The formation of DNA recombinants from cellular DNAs that have been enriched[5] by physical and chemical techniques (i.e., not by cloning) and

which are free of harmful genes can be carried out under lower containment conditions than used for the corresponding shotgun experiment. In general, the containment can be decreased one step in physical containment (P4 → P3 → P2 → P1) while maintaining the biological containment specified for the shotgun experiment, or one step in biological containment (EK3 → EK2 → EK1) while maintaining the specified physical containment--provided that the new condition is not less than that specified above for characterized clones from shotgun experiments (Section <a>--iii).

<c> Plasmids, bacteriophages, and other viruses

Recombinants formed between EK-type vectors and other plasmid or virus DNAs have in common the potential for acting as double vectors because of the replication functions in these DNAs. The containment conditions given below apply only to propagation of the DNA recombinants in E. coli K-12 hosts. They do not apply to other hosts where they may be able to replicate as a result of functions provided by the DNA inserted into the EK vectors. These are considered under other host-vector systems.

(i) Animal viruses

P4 + EK2 or P3 + EK3 shall be used to isolate DNA recombinants that include all or part of the genome of an animal virus. This recommendation applies not only to experiments of the "shotgun" type but also to those involving partially characterized subgenomic segments of viral DNAs (for example, the genome of defective viruses, DNA fragments isolated after treatment of viral genomes with restriction enzymes, etc). When cloned recombinants have been shown by suitable biochemical and biological

tests to be free of harmful regions, they can be handled in P3 + EK2 conditions. In the case of DNA viruses, harmless regions include the late region of the genome; in the case of DNA copies of RNA viruses, they might include the genes coding for capsid proteins or envelope proteins.

(ii) Plant viruses

P3 + EK1 or P2 + EK2 conditions shall be used to form DNA recombinants that include all or part of the genome of a plant virus.

(iii) Eukaryotic organelle DNAs

The containment conditions given below apply only when the organelle DNA has been purified[6] from isolated organelles. Mitochondrial DNA from primates: P3 + EK1 or P2 + EK2. Mitochondrial or chloroplast DNA from other eukaryotes: P2 + EK1. Otherwise, the conditions given under shotgun experiments apply.

(iv) Prokaryotic plasmid and phage DNAs

Plasmids and phage from hosts that exchange genetic information with E. coli - Experiments with DNA recombinants formed from plasmids or phage genomes that have not been characterized with regard to presence of harmful genes or are known to contribute significantly to the pathogenicity of their normal hosts must use the containment conditions specified for shotgun experiments with DNAs from the respective host. If the DNA recombinants are formed from plasmids or phage that are known not to contain harmful genes, or from purified[6] and characterized plasmid or phage DNA segments known not to contain harmful genes, the experiments can be performed with P1 physical containment + an EK1 host-vector.

Plasmids and phage from hosts that do not exchange genetic information with E. coli - The rules for shotgun experiments with DNA from the host apply to their plasmids or phages. The minimum containment conditions for this category (P2 + EK2, or P3 + EK1) can be used for plasmid and phage, or for purified[6] and characterized segments of plasmid and phage DNAs, when the risk that the recombinant DNAs will increase the pathogenicity or ecological potential of the host is judged to be minimal.

NOTE: Where applicable, cDNAs (i.e., complementary DNAs) synthesized in vitro from cellular or viral RNAs are included within each of the above classifications. For example, cDNAs formed from cellular RNAs that are not purified and characterized are included under <a>, shotgun experiments; cDNAs formed from purified and characterized RNAs are included under <b>; cDNAs formed from viral RNAs are included under <c>; etc.

3. Experiments with other prokaryotic host-vectors

Other prokaryotic host-vector systems are at the speculative, planning, or developmental stage, and consequently do not warrant detailed treatment here at this time. However, the containment criteria for different types of DNA recombinants formed with E. coli K-12 host-vectors can, with the aid of some general principles given here, serve as a guide for containment conditions with other host-vectors when appropriate adjustment is made for their different habitats and characteristics. The newly developed host-vector systems

should offer some distinct advantage over the E. coli K-12 host-vectors--for instance, thermophilic organisms or other host-vectors whose major habitats do not include humans and/or economically important animals and plants. In general, the strain of any prokaryotic species used as the host is to conform to the definition of Class 1 etiologic agents given in ref. 5 (i.e., "Agents of no or minimal hazard...."), and the plasmid or phage vector should not make the host more hazardous. Appendix A gives a detailed discussion of the B. subtilis system, the most promising alternative to date.

At the initial stage, the host-vector must exhibit at least a moderate level of biological containment comparable to EK1 systems, and should be capable of modification to obtain high levels of containment comparable to EK2 and EK3. The type of confirmation test(s) required to move a host-vector from an EK2-type classification to an EK3-type will clearly depend upon the preponderant habitat of the host-vector. For example, if the unmodified host-vector propagates mostly in, on, or around higher plants, but not appreciably in warm-blooded animals, modification should be designed to reduce the probability that the host-vector can escape to and propagate in, on, or around such plants, or transmit recombinant DNA to other bacterial hosts that are able to occupy these ecological niches, and it is these lower probabilities which must be confirmed. The following principles are to be followed in using the containment criteria given for experiments with E. coli K-12 host-vectors as a guide for other prokaryotic systems. Experiments with DNA from prokaryotes (and their plasmids or viruses) are classified according to whether the prokaryote in question exchanges

genetic information with the host-vector or not, and the containment conditions given for these two classes with E. coli K-12 host-vectors applied. Experiments with recombinants between plasmid or phage vectors and DNA that extends the range of resistance of the recipient species to therapeutically useful drugs must use P3 physical containment + a host-vector comparable to EK1 or P2 physical containment + a host-vector comparable to EK2. Transfer of recombinant DNA to plant pathogens can be made safer by using nonreverting, doubly auxotrophic, non-pathogenic variants. Experiments using a plant pathogen that affects an element of the local flora will require more stringent containment than if carried out in areas where the host plant is not common.

Experiments with DNAs from eukaryotes (and their plasmids or viruses) can also follow the criteria for the corresponding experiments with E. coli K-12 vectors if the major habitats of the given host-vector overlap those of E. coli. If the host-vector has a major habitat that does not overlap those of E. coli (e.g., root nodules in plants), then the containment conditions for some eukaryotic recombinant DNAs need to be increased (for instance, higher plants and their viruses in the preceding example), while others can be reduced.

4. Experiments with eukaryotic host-vectors

<a> Animal host-vector systems - Because host cell lines generally have little if any capacity for propagation outside the laboratory, the primary focus for containment is the vector, although cells should also be derived from cultures expected to be of minimal hazard. Given good microbiological practices, the most likely mode of escape of recombinant DNAs from a physically contained laboratory is carriage by humans; thus vectors

should be chosen that have little or no ability to replicate in human cells. To be used as a vector in a eukaryotic host, a DNA molecule needs to display all of the following properties:

(1) It shall not consist of the whole genome of any agent that is infectious for humans or that replicates to a significant extent in human cells in tissue culture.

(2) Its functional anatomy should be known--that is, there should be a clear idea of the location within the molecule of:

a) the sites at which DNA synthesis originates and terminates,

b) the sites that are cleaved by restriction endonucleases,

c) the template regions for the major gene products.

(3) It should be well studied genetically. It is desirable that mutants be available in adequate number and variety, and that quantitative studies of recombination have been performed.

(4) The recombinant must be defective, that is, its propagation as a virus is dependent upon the presence of a complementing helper genome. This helper should either (a) be integrated into the genome of a stable line of host cells (a situation that would effectively limit the growth of the vector to that particular cell line) or (b) consist of a defective genome or an appropriate conditional lethal mutant virus (in which case the experiments would be done under non-permissive conditions), making vector and helper dependent upon each other for propagation. However, if

none of these is available, the use of a non-defective genome as helper would be acceptable.

Currently only two viral DNAs can be considered as meeting these requirements: these are the genomes of polyoma virus and SV40.

Of these, polyoma virus is highly to be preferred. SV40 is known to propagate in human cells, both *in vivo* and *in vitro*, and to infect laboratory personnel, as evidenced by the frequency of their conversion to producing SV40 antibodies. Also, SV40 and related viruses have been found in association with certain human neurological and malignant diseases. SV40 shares many properties, and gives complementation, with the common human papova viruses. By contrast, there is no evidence that polyoma infects humans, nor does it replicate to any significant extent in human cells *in vitro*. However, this system still needs to be studied more extensively. Appendix B gives further details and documentation.

Taking account of all these factors:

1. Polyoma Virus

   a Recombinant DNA molecules consisting of defective polyoma virus genomes plus DNA sequences of any non-pathogenic organism, including Class 1 viruses (5), can be propagated in or used to transform cultured cells. P3 conditions are required. Appropriate helper virus can be used if needed. Whenever there is a choice, it is urged

that mouse cells, derived preferably from embryos, be used as the source of eukaryotic DNA. Polyoma virus is a mouse virus and recombinant DNA molecules containing both viral and cellular sequences are already known to be present in virus stocks grown at a high multiplicity. Thus, recombinants formed *in vitro* between polyoma virus DNA and mouse DNA are presumably not novel from an evolutionary point of view.

b. Such experiments are to be done under P4 conditions if the recombinant DNA contains segments of the genomes of Class 2 animal viruses (5). Once it has been shown by suitable biochemical and biological tests that the cloned recombinant contains only harmless regions of the viral genome (see Section IIIB-2-c-i) and that the host range of the polyoma virus vector has not been altered, experiments can be continued under P3 conditions.

2. SV40 Virus

a. Defective SV40 genomes, with appropriate helper, can be used as a vector for recombinant DNA molecules containing sequences of any non-pathogenic organism or Class I virus (5), (i.e., a shotgun type experiment). P4 conditions are required. Established lines of cultured cells should be used.

b. Such experiments are to be carried out in P3 (or P4) conditions if the non-SV40 DNA segment is (a) a purified[6] segment of prokaryotic DNA lacking toxigenic genes, or (b) a segment

of eukaryotic DNA whose function has been established, which does not code for a toxic product, and which has been previously cloned in a prokaryotic host-vector system. It shall be confirmed that the defective virus - helper virus system does not replicate significantly more efficiently in human cells in tissue culture than does SV40, following infection at a multiplicity of infection of one or more helper SV40 viruses per cell.

c. A recombinant DNA molecule consisting of defective SV40 DNA lacking substantial segments of the late region, plus DNA from non-pathogenic organisms or Class I viruses (5), can be propagated as an autonomous cellular element in established lines of cells under P3 conditions provided that there is no exogenous or endogenous helper, and that it is demonstrated that no infectious virus particles are being produced. Until this has been demonstrated, the appropriate containment conditions specified in 2. a. and 2. b. shall be used.

d. Recombinant DNA molecules consisting of defective SV40 DNA and sequences from non-pathogenic prokaryotic or eukaryotic organisms or Class I viruses (5) can be used to transform established lines of non-permissive cells under P3 conditions. It must be demonstrated that no infectious virus particles are being produced; rescue

of SV40 from such transformed cells by co-cultivation or transfection techniques must be carried out in P4 conditions.

3. Efforts are to be made to ensure that all cell lines are free of virus particles and mycoplasma.

Since SV40 and polyoma are limited in their scope to act as vectors, chiefly because the amount of foreign DNA that the normal virions can carry probably cannot exceed 2×10^6 daltons, the development of systems in which recombinants can be cloned and propagated purely in the form of DNA, rather than in the coats of infectious agents is necessary. Plasmid forms of viral genomes or organelle DNA need to be explored as possible cloning vehicles in eukaryotic cells.

<b> Plant host-vector systems

For cells in tissue cultures, seedlings, or plant parts (e.g. tubers, stems, fruits, and detached leaves) or whole mature plants of small species (e.g., Arabidopsis) the P1-P4 containment conditions that we have specified previously are relevant concepts. However, work with most plants poses additional problems. The greenhouse facilities accompanying P2 laboratory physical containment conditions can be provided by: (i) insect-proof greenhouses, (ii) appropriate sterilization of contaminated plants, pots, soil, and runoff water, and (iii) adoption of the other standard practices for microbiological work. P3 physical containment can be sufficiently approximated by confining the operations with whole plants to growth chambers like those used for work with radioactive isotopes,

provided that (i) such chambers are modified to produce a negative pressure environment with the exhaust air appropriately filtered, (ii) that other operations with infectious materials are carried out under the specified P3 conditions, and (iii) to guard against inadvertent insect transmission of recombinant DNA, growth chambers are to be routinely fumigated and only used in insect proof rooms. The P2 and P3 conditions specified earlier are therefore extended to include these cases for work on higher plants.

The host cells for experiments on recombinant DNAs may be cells in culture, in seedling or plant parts. Whole plants or plant parts that cannot be adequately contained shall not be used as hosts for shotgun experiments at this time, and attempts to infect whole plants with recombinant DNA shall not be initiated until the effects on host cells in culture, seedlings or plant parts have been thoroughly studied.

Organelle or plasmid DNAs or DNAs of viruses of restricted host range may be used as vectors. In general, similar criteria for selecting host-vectors to those given in the preceding section on animal systems are to apply to plant systems.

DNA recombinants formed between the initial moderately contained vectors and DNA from cells of species in which the vector DNA can replicate, require P2 physical containment. However, if the source of the DNA is itself pathogenic or known to carry pathogenic agents, or to produce products dangerous to plants, or if the vector is an unmodified virus of unrestricted host range, the experiments shall be carried out under P3 conditions.

Experiments on recombinant DNAs formed between the above vectors and DNAs from other species can also be carried out under P2 if that DNA has been purified[6] and determined not to contain harmful genes. Other-

wise, the experiments shall be carried out under P3 conditions if the source of the inserted DNA is not itself a pathogen, or known to carry such pathogenic agents, or to produce harmful products--and under P4 conditions if these conditions are not met.

The development and use of host-vector systems that exhibit a high level of biological containment permit a decrease of one step in the physical containment specified above (P4 → P3 → P2 → P1).

<c> Fungal or similar lower eukaryotic host-vector systems

The containment criteria for experiments on recombinant DNAs using these host-vectors most closely resemble those for prokaryotes, rather than those for the preceding eukaryotes, in that the host cells usually exhibit a capacity for dissemination outside the laboratory that is similar to that for bacteria. We therefore consider that the containment guidelines given for experiments with E. coli K-12 and other prokaryotic host-vectors (Sections IIIB-1 and -2, respectively) provide adequate direction for experiments with these lower eukaryotic host-vectors. This is particularly true at this time since the development of these host-vectors is presently in the speculative stage.

IV. Roles and Responsibilities

Safety in research involving recombinant DNA molecules depends upon how the research team applies these guidelines. Motivation and critical judgment are necessary, in addition to specific safety knowledge, to ensure protection of personnel, the public, and the environment.

The guidelines given here are to help the principal investigator determine the nature of the safeguards that should be implemented. These guidelines will be incomplete in some respects because all conceivable experiments with recombinant DNAs cannot now be anticipated. Therefore, they cannot substitute for the investigator's own knowledgeable and discriminating evaluation. Whenever this evaluation calls for an increase in containment over that indicated in the guidelines, the investigator has a responsibility to institute such an increase. In contrast, the containment conditions called for in the guidelines should not be decreased without review and approval at the institutional and NIH levels.

The following roles and responsibilities define an administrative framework in which safety is an essential and integrated function of research involving recombinant DNA molecules.

A. Principal Investigator - The principal investigator has the primary responsibility for: (i) determining the real and potential biohazards of the proposed research, (ii) determining the appropriate level of biological and physical containment, (iii) selecting the microbiological practices and laboratory techniques for handling recombinant DNA materials, (iv) preparing procedures for dealing with accidental spills and overt personnel contamination, (v) determining the applicability of various

precautionary medical practises, serological monitoring, and immunization, when available, (vi) securing approval of the proposed research prior to initiation of work, (vii) submitting information on purported EK2 and EK3 systems to the NIH Recombinant DNA Molecule Program Advisory Committee and making the strains available to others, (viii) reporting to the institutional biohazards committee and the NIH Office of Recombinant DNA Activities new information bearing on the guidelines, such as technical information relating to hazards and new safety procedures or innovations, (ix) applying for approval from the NIH Recombinant DNA Molecule Program Advisory Committee for large scale experiments with recombinant DNAs known to make harmful products (i.e., more than 10 liters of culture), and (x) applying to NIH for approval to lower containment levels when a cloned DNA recombinant derived from a shotgun experiment has been rigorously characterized and there is sufficient evidence that it is free of harmful genes.

Before work is begun, the principal investigator is responsible for: (i) making available to program and support staff copies of those portions of the approved grant application that describe the biohazards and the precautions to be taken, (ii) advising the program and support staff of the nature and assessment of the real and potential biohazards, (iii) instructing and training this staff in the practices and techniques required to ensure safety, and in the procedures for dealing with accidentally created biohazards, and (iv) informing the staff of the reasons and provisions for any advised or requested precautionary medical practises, vaccinations, or serum collection.

During the conduct of the research, the principal investigator is responsible for: (i) supervising the safety performance of the staff to ensure that the required safety practices and techniques are employed, (ii) investigating and reporting in writing to the NIH Office of Recombinant DNA Activities and the institutional biohazards committee any serious or extended illness of a worker or any accident that results in (a) inoculation of recombinant DNA materials through cutaneous penetration, (b) ingestion of recombinant DNA materials, (c) probable inhalation of recombinant DNA materials following gross aerosolization, or (d) any incident causing serious exposure to personnel or danger of environmental contamination, (iii) investigating and reporting in writing to the NIH Office of Recombinant DNA Activities and the institutional biohazards committee any problems pertaining to operation and implementation of biological and physical containment safety practices and procedures, or equipment or facility failure, (iv) correcting work errors and conditions that may result in the release of recombinant DNA materials, and (v) ensuring the integrity of the physical containment (e.g., biological safety cabinets) and the biological containment (e.g., genotypic and phenotypic characteristics, purity, etc.).

B. Institution - Since in almost all cases, NIH grants are made to institutions rather than to individuals, all the responsibilities of the principal investigator listed above are the responsibilities of the institution under the grant, fulfilled on its behalf by the principal investigator. In addition, the institution is responsible for establishing an institutional biohazards committee[7] to: (i) advise the institution on policies, (ii) create and maintain a central reference file and library of catalogs, books,

articles, newsletters, and other communications as a source of advice and reference regarding, for example, the availability and quality of the safety equipment, the availability and level of biological containment for various host-vector systems, suitable training of personnel and data on the potential biohazards associated with certain recombinant DNAs, (iii) develop a safety and operations manual for any P4 facility maintained by the institution and used in support of recombinant DNA research, (iv) certify to the NIH on applications for research support and annually thereafter, that facilities, procedures, and practices and the training and expertise of the personnel involved have been reviewed and approved by the institutional biohazards committee.

The biohazards committee must be sufficiently qualified through the experience and expertise of its membership and the diversity of its membership to ensure respect for its advice and counsel. Its membership should include individuals from the institution or consultants, selected so as to provide a diversity of disciplines relevant to recombinant DNA technology, biological safety, and engineering. In addition to possessing the professional competence necessary to assess and review specific activities and facilities, the committee should possess or have available to it, the competence to determine the acceptability of its findings in terms of applicable laws, regulations, standards of practices, community attitudes, and health and environmental considerations. Minutes of the meetings should be kept and made available for public inspection. The institution is responsible for reporting names of and relevant background information on the members of its biohazards committee to the NIH.

C. NIH Initial Review Groups (Study Sections) - The NIH Study Sections, in addition to reviewing the scientific merit of each grant application involving recombinant DNA molecules, are responsible for: (i) making an independent evaluation of the real and potential biohazards of the proposed research on the basis of these guidelines, (ii) determining whether the proposed physical containment safeguards certified by the institutional biohazards committee are appropriate for control of these biohazards, (iii) determining whether the proposed biological containment safeguards are appropriate, (iv) referring to the NIH Recombinant DNA Molecule Program Advisory Committee or the NIH Office of Recombinant DNA Activities those problems pertaining to assessment of biohazards or safeguard determination that cannot be resolved by the Study Sections.

The membership of the Study Sections will be selected in the usual manner. Biological safety expertise, however, will be available to the Study Sections for consultation and guidance.

D. NIH Recombinant DNA Molecule Program Advisory Committee - The Recombinant DNA Molecule Program Advisory Committee advises the Secretary, Department of Health, Education, and Welfare, the Assistant Secretary for Health, Department of Health, Education, and Welfare, and the Director, National Institutes of Health, on a program for the evaluation of potential biological and ecological hazards of recombinant DNAs (molecules resulting from different segments of DNA that have been joined together in cell-free systems, and which have the capacity to infect and replicate in some host cell, either autonomously or as an integrated part of their host's genome), on the development of procedures which are designed to prevent the spread of such

molecules within human and other populations, and on guidelines to be followed by investigators working with potentially hazardous recombinants.

The NIH Recombinant DNA Molecule Program Advisory Committee has responsibility for: (i) revising and updating guidelines to be followed by investigators working with DNA recombinants, (ii) for the time being, receiving information on purported EK2 and EK3 systems and evaluating and certifying that host-vector systems meet EK2 or EK3 criteria, (iii) resolving questions concerning potential biohazard and adequacy of containment capability if NIH staff or NIH Initial Review Group so request, and (iv) reviewing and approving large scale experiments with recombinant DNAs known to make harmful products (e.g., more than 10 liters of culture).

E. NIH Staff - NIH Staff has responsibility for: (i) assuring that no NIH grants or contracts are awarded for DNA recombinant research unless they (a) conform to these guidelines, (b) have been properly reviewed and recommended for approval, and (c) include a properly executed Memorandum of Understanding and Agreement, (ii) reviewing and responding to questions or problems or reports submitted by institutional biohazards committees or principal investigators, and disseminating findings, as appropriate, (iii) receiving and reviewing applications for approval to lower containment levels when a cloned DNA recombinant derived from a shotgun experiment has been rigorously characterized and there is sufficient evidence that it is free of harmful genes, (iv) referring items covered under (ii) and (iii) above to the NIH Recombinant DNA Molecule Program Advisory Committee, as deemed necessary, and (v) performing site inspections of all P4 physical containment facilities, engaged in DNA recombinant research, and of other facilities as deemed necessary.

V. Footnotes

[1]Biological Safety Cabinets referred to in this section are classified as Class I, Class II or Class III cabinets. A Class I cabinet is a ventilated cabinet for personnel protection having an inward flow of air away from the operator. The exhaust air from this cabinet is filtered through a high efficiency or high efficiency particulate air (HEPA) filter before being discharged to the outside atmosphere. This cabinet is used in three operational modes; (1) with an 8 inch high full width open front, (2) with an installed front closure panel (having four eight inch diameter openings) without gloves, and (3) with an installed front closure panel equipped with arm length rubber gloves. The face velocity of the inward flow of air through the full width open front is 75 feet per minute or greater. A Class II cabinet is a ventilated cabinet for personnel and product protection having an open front with inward air flow for personnel protection, and HEPA filtered mass recirculated air flow for product protection. The cabinet exhaust air is filtered through a HEPA filter. The face velocity of the inward flow of air through the full width open front is 75 feet per minute or greater. Design and performance specifications for Class II cabinets have been adopted by the National Sanitation Foundation, Ann Arbor, Michigan. A Class III cabinet is a closed front ventilated cabinet of gas tight construction which provides the highest level of personnel protection of all Biohazard Safety Cabinets. The interior of the cabinet is protected from contaminants exterior to the cabinet. The cabinet is fitted with arm length rubber gloves and is operated under a negative pressure of at least 0.5 inches water gauge. All supply air is filtered through HEPA filters. Exhaust air is filtered through HEPA filters or incinerated before being discharged to the outside environment.

[2]Defined as observable under optimal laboratory conditions by transformation, transduction, phage infection and/or conjugation with transfer of phage, plasmid and/or chromosomal genetic information.

[3]The bacteria which constitute Class 2 of ref. 5 ("Agents of ordinary potential hazard....") represent a broad spectrum of etiologic agents which possess different levels of virulence and degrees of communicability. We think it appropriate for our specific purpose to further subdivide the agents of Class 2 into those which we believe to be of relatively low pathogenicity and those which are moderately pathogenic. The several specific examples given may suffice to illustrate the principle.

[4]The terms "characterized" and "free of harmful genes" are unavoidably vague. But, in this instance, before containment conditions lower than the ones used to clone the DNA can be adopted, the investigator must obtain approval from the National Institutes of Health. Such approval would be contingent upon data concerning: (a) the absence of potentially harmful genes (e.g., sequences contained in indigenous tumor viruses or which code for toxic substances), (b) the relation between the recovered and desired segment (e.g., hybridization and restriction endonuclease fragmentation analysis where applicable), and (c) maintenance of the biological properties of the vector.

[5] A DNA preparation is defined as enriched if the desired DNA represents at least 99% (w/w) of the total DNA in the preparation. The reason for lowering the containment level when this degree of enrichment has been obtained is based on the fact that the total number of clones that must be examined to obtain the desired clone is markedly reduced. Thus, the probability of cloning a harmful gene could, for example, be reduced by more that 10^5-fold when a nonrepetitive gene from mammals was being sought. Furthermore, the level of purity specified here makes it easier to establish that the desired DNA does not contain harmful genes.

[6] The DNA preparation is defined as purified if the desired DNA represents at least 99% (w/w) of the total DNA in the preparation, provided that it was verified by more than one procedure.

[7] In special circumstances, in consultation with the NIH Office of Recombinant DNA Activities, an area biohazards committee may be formed, composed of members from the institution and/or other organizations beyond its own staff, as an alternative when additional expertise outside the institution is needed for the indicated reviews.

VI. References

1. Berg, P., D. Baltimore, H.W. Boyer, S.N. Cohen, R.W. Davis, D.S. Hogness, D. Nathans, R.O. Roblin, J.D. Watson, S. Weissman, and N.D. Zinder (1974). Potential Biohazards of Recombinant DNA Molecules. Science 185, 303.

2. Advisory Board for the Research Councils. Report of a Working Party on the Experimental Manipulation of the Genetic Composition of Micro-Organisms. Presented to Parliament by the Secretary of State for Education and Science by Command of Her Majesty. January, 1975. London: Her Majesty's Stationery Office, 1975.

3. Berg, P., D. Baltimore, S. Brenner, R.O. Roblin and M.F. Singer (1975). Summary Statement of the Asilomar Conference on Recombinant DNA Molecules. Science 188, 991; Nature 225, 442; Proc. Nat. Acad. Sci. 72, 1981.

4. Laboratory Safety at the Center for Disease Control (Sept., 1974). U.S. Department of Health, Education and Welfare Publication No. CDC 75-8118.

5. Classification of Etiologic Agents on the Basis of Hazard. (4th Edition, July, 1974). U.S. Department of Health, Education and Welfare. Public Health Service. Center for Disease Control, Office of Biosafety, Atlanta, Georgia 30333.

6. National Cancer Institute Safety Standards for Research Involving Oncogenic Viruses (Oct., 1974). U.S. Department of Health, Education and Welfare Publication No. (NIH) 75-790.

7. National Institutes of Health Biohazards Safety Guide (1974). U.S. Department of Health, Education and Welfare. Public Health Service, National Institutes of Health. U.S. Government Printing Office Stock No. 1740-00383.

8. Biohazards in Biological Research (1973). A. Hellman, M.N. Oxman and R. Pollack (ed.). Cold Spring Harbor Laboratory.

9. Handbook of Laboratory Safety (1971; 2nd Edition). N.V. Steere (ed.). The Chemical Rubber Co., Cleveland.

10. Bodily, H. L. (1970). General Administration of the Laboratory. H. L. Bodily, E. L. Updyke and J. O. Mason (eds.), Diagnostic Procedures for Bacterial, Mycotic and Parasitic Infections. American Public Health Association, New York. pp.11-28.

11. Darlow, H.M. (1969). Safety in the Microbiological Laboratory. In J.R. Norris and D.W. Robbins (ed.), Methods in Microbiology. Academic Press, Inc. New York. pp. 169-204.

12. The Prevention of Laboratory Acquired Infection (1974). C.H. Collins, E. G. Hartley, and R. Pilsworth, Public Health Laboratory Service, Monograph Series No. 6.

13. Chatigny, M. A. (1961). Protection Against Infection in the Microbiological Laboratory: Devices and Procedures. In W. W. Umbreit (ed.). Advances in Applied Microbiology. Academic Press, New York, N.Y. 3:131-192.

14. Design Criteria for Viral Oncology Research Facilities, U.S. Department of Health, Education and Welfare, Public Health Service, National Institutes of Health, DHEW Publication No. (NIH) 75-891, 1975.

15. Kuehne, R. W. (1973). Biological Containment Facility for Studying Infectious Disease. Appl. Microbiol. 26:239-243.

16. Runkle, R. S. and G. B. Phillips.(1969). Microbial Containment Control Facilities. Van Nostrand Reinhold, New York.

17. Chatigny, M. A. and D. I. Clinger (1969). Contamination Control in Aerobiology. In R. L. Dimmick and A. B. Akers (eds.). An Introduction to Experimental Aerobiology. John Wiley & Sons, New York, pp. 194-263.

18. Grunstein, M. and D.S. Hogness (1975). Colony Hybridization: A Method for the Isolation of Cloned DNAs That Contain a Specific Gene. Proc. Nat. Acad. Sci. U.S.A. 72, 3961-3965.

19. Morrow, J.F., S.N. Cohen, A.C.Y. Chang, H.W. Boyer, H.M. Goodman and R.B. Helling (1974). Replication and Transcription of Eukaryotic DNA in Escherichia coli. Proc. Nat. Acad. Sci. USA 71, 1743-1747.

20. Hershfield, V., H.W. Boyer, C. Yanofsky, M.A. Lovett and D.R. Helinski (1974). Plasmid ColE1 as a Molecular Vehicle for Cloning and Amplification of DNA. Proc. Nat. Acad. Sci. USA 71, 3455-3459.

21. Wensink, P.C., D.J. Finnegan, J.E. Donelson, and D.S. Hogness (1974). A System for Mapping DNA Sequences in the Chromosomes of Drosophila melanogaster. Cell 3, 315-325.

22. Timmis, K., F. Cabello and S.N. Cohen (1974). Utilization of Two Distinct Modes of Replication by a Hybrid Plasmid Constructed In Vitro from Separate Replicons. Proc. Nat. Acad. Sci. USA 71, 4556-4560.

23. Glover, D.M., R.L. White, D.J. Finnegan and D.S. Hogness (1975). Characterization of Six Cloned DNAs from Drosophila melanogaster. Including one that Contains the Genes for rRNA. Cell 5, 149-155.

24. Kedes, L.H., A.C.Y. Chang, D. Houseman and S.N. Cohen (1975). Isolation of Histone Genes from Unfractionated Sea Urchin DNA by Subculture Cloning in E. coli. Nature 255, 533.

25. Tanaka, T. and B. Weisblum (1975). Construction of a Colicin E1-R Factor Composite Plasmid In Vitro: Means for Amplification of Deoxyribonucleic Acid. J. Bacteriol. 121, 354-362.

26. Tanaka, T., B. Weisblum, M. Schnoss and R. Inman (1975). Construction and Characterization of a Chimeric Plasmid Composed of DNA from Escherichia coli and Drosophila melanogaster. Biochemistry 14, 2064-2072.

27. Thomas, M., J.R. Cameron and R.W. Davis (1974). Viable Molecular Hybrids of Bacteriophage Lambda and Eukaryotic DNA. Proc. Nat. Acad. Sci. USA 71, 4579-4583.

28. Murray, N.E. and K. Murray (1974). Manipulation of Restriction Targets in Phage λ to form Receptor Chromosomes for DNA Fragments. Nature 251, 476-481.

29. Rambach, A. and P. Tiollais (1974). Bacteriophage λ Having EcoR1 Endonuclease Sites only in the Non-essential Region of the Genome. Proc. Nat. Acad. Sci. USA 71, 3927-3930.

30. Smith, H.W. (1975) Survival of Orally-Administered Escherichia coli K12 in the Alimentary Tract of Man. Nature 255, 500-502.

31. Anderson, E.S. (1975). Viability of, and Transfer of a Plasmid from Escherichia coli K12 in the human intestine. Nature 255, 502-504.

32. Falkow, S. (1975). Unpublished experiments quoted in Appendix D of the Report of the Organizing Committee of the Asilomar Conference on Recombinant DNA Molecules (P. Berg, D. Baltimore, S. Brenner, R.O. Roblin and M. Singer, eds.) submitted to the National Academy of Sciences.

33. R. Curtiss III, personal communication.

34. Novick, R.P. and S.I. Morse (1967). In Vivo Transmission of Drug Resistance Factors between Strains of Staphylococcus aureus.J. Exp. Med. 125, 45-59.

35. Anderson, J.D., W.A. Gillespie and M.H. Richmond. 1974. Chemotherapy and Antibiotic Resistance Transfer between Enterobacteria in the Human Gastrointestinal Tract. J. Med. Microbiol. 6, 461-473.

36. Ronald Davis, personal communication.

37. K. Murray, personal communication; W. Szybalski, personal communication.

38. Manly, K.R., E.R. Signer and C.M. Radding (1969). Nonessential Functions of Bacteriophage λ. Virology 37 177.

39. Gottesman, M.E. and R.A. Weisberg (1971). Prophage Insertion and Excision. In The Bacteriophage Lambda (A.D. Hershey, ed.). Cold Spring Harbor Laboratory pp. 113-138.

40. Shimada, K., R.A. Weisberg and M.E. Gottesman (1972). Prophage Lambda at Unusual Chromosomal Locations: I. Location of the Secondary Attachment Sites and the Properties of the Lysogens. J. Mol. Biol. 63, 483-503.

41. Signer, E. (1969). Plasmid Formation: A New Mode of Lysogeny by Phage λ. Nature 223, 158-160.

42. Adams, M.H. (1959). Bacteriophages. Intersciences Publishers, Inc., New York.

43. Jacob, F. and E.L. Wollman. 1956). Sur les Processus de Conjugaison et de Recombinasion chez Escherichia coli. I. L'induction par Conjugaison ou Induction Zygotique. Ann. Inst. Pasteur 91, 486-510.

44. J.S. Parkinson as cited (p. 8) by Hershey, A.D. and W. Dove (1971). Introduction to Lambda. In: The Bacteriophage λ. A.D. Hershey, ed. Cold Spring Harbor Laboratory, New York.

Statement on the use of *Bacillus subtilis* in recombinant molecule technology

Unquestionably, Escherichia coli is the most well characterized unicellular organism. Years of basic research have enabled investigators to develop a well characterized genetic map, to obtain detailed knowledge of virulent and temperate bacteriophages, and to explore the physiology, genetics, and regulation of plasmids. More recently, the development of DNA-mediated transformation has permitted exogenous fragments or molecules of DNA to be incorporated into the genome or to reside as self-replicating units. The discovery of transformation of Bacillus subtilis by Spizizen (1) stimulated the development of an alternative model system. The purpose of this report is to summarize the current status of this genetic system and to describe the actual and potential vectors and vehicles available for recombinant molecule technology.

A. Current knowledge of the chomosomal architecture and mechanisms of genetic exchange in B. *subtilis*

Two mechanisms of genetic exchange have been utilized to establish the linkage map of B. subtilis, DNA-mediated transformation (capable of transferring approximately 1% of the genome) and transduction with bacteriophage PBS1 (capable of transferring 5-8% of the chromosome). Recent detailed genetic studies with PBS1 by Lepesant-Kejzlorovà et al. (2) have resulted in the development of a circular genetic map for this organism. The current edition of the map (3) contains 196 loci. Biophysical analyses

have established that the chromosome is circular (4) and replicates bidirectionally (5).

Transformation with purified fragments of DNA is a highly efficient process in B. subtilis with frequencies of 1 to 4% usually attained for any auxotrophic or antibiotic resistance markers. Frequencies of approximately 10% transformation can be achieved with DNA prepared from gently lysed L-forms or protoplasts (6). These large fragments of DNA are readily incorporated by the recipient cell. Generalized transduction occurs with bacteriophages SP10 (7), PBS1 (8), and SPP1 (9), while a low frequency of specialized transduction has been reported with bacteriophage ϕ105 (10).

Although transformation is most efficient in homologous crosses (B. subtilis into B. subtilis), it has also been possible to exchange DNA among closely related species (11). The most extensively studied members of the B. subtilis genospecies include B. licheniformis, B. pumilus, B. amyloliquefaciens, and B. globigii (refer to reference 12 for a review and references 13-15 for examples of this heterologous exchange). This exchange occurs even though there is a surprisingly wide discrepancy between DNA - DNA hybridization among these organisms (16). Even though the frequency of transformation is low in the heterologous cross [e.g., B. amyloliquefaciens (donor)/B subtilis (recipient)], the newly acquired DNA from B. amyloliquefaciens in the B. subtilis background can be readily transferred at high efficiencies to other recipient strains of B. subtilis (14). Therefore, the extremely high frequency of transformation permits the recognition and selection of rare events.

B. Current and potential vectors for recombinant molecule experiments

Lovett and coworkers have recently described cryptic plasmids in B. pumilus (17) and B. subtilis (18). Of these organisms, B. subtilis ATCC 7003 appears to be the most useful since it carries one to two copies of a plasmid with a molecular weight of 46×10^6. This strain is also closely related to B. subtilis 168. Another strain of B. subtilis (ATCC 15841) contains 16 copies of a plasmid with a molecular weight of 4.6×10^6. Currently it is not known whether genetic markers can be readily introduced into these plasmids. To date it has not been possible to readily stabilize plasmids derived from B. pumilus in B. subtilis even with heavy selective pressure (P. Lovett, personal communication).

Two temperate bacteriophages are under development as vectors in B. subtilis, ϕ3T and SP02. Lysogeny of thymine auxotrophs (strains carrying thyA thyB) by bacteriophage ϕ3T results in "conversion" to a Thy^+ phenotype. The attachment site for this bacteriophage and the bacteriophage gene for thymidylate synthetase (thyP) map between the bacterial thyA and thyB loci in the terminal region of the chromosome of B. subtilis (19). The viral genome is readily cleaved by the site-specific endonuclease, Bam 1 (20), to produce 5 fragments (one of which carries the thyP gene). The thyP carrying gene can be integrated into the bacterial genome in the absence of the intact viral genome. Because deletions are available that include the thyP region, it is theoretically possible to introduce thyP at many sites on the chromosome. The thyP gene can be readily purified

for insertion into plasmids or utilized as a scaffold to integrate other heterologous DNA into the chromosome of B. subtilis. Alternatively, it is possible to purify fragments of the chromosome by gel electrophoresis (21, 22), for insertion into bacteriophage ϕ3T or SPO2. At present, unfortunately, only the former carries a selective marker, i.e., the gene for thymidylate synthetase, thyP.

C. Development of vehicles

B. subtilis is a Gram-positive sporulating rod that usually inhabits soil. Although it can exist on cutaneous surfaces of man (23) and experimental animals, it rarely produces disease. To develop a suitable vehicle it is imperative to have a host that is asporogenic. The most appropriate deletion mutation is deletion 29 (cit D). In addition to a deficiency in sporulation this mutant rapidly lyses when it has reached the end of its growth cycle. Presumably this is due to the failure to inactivate one of the autolytic enzymes (24). Through the introduction of a D-alanine requirement (34 ug/ml) it is possible to block transport of compounds that are transported by active transport (25,26). The further introduction of thymine auxotrophy (defects in the thyA thyB loci) will enable the strain to survive only with a plasmid vector carrying the purified thyP gene from bacteriophage ϕ3T or a defective bacteriophage ϕ3T carrying the thyP gene but attached to the chromosome at an alternative site (due to the presence of deletion 29 in the host). We have recently isolated temperature-sensitive thyP mutants. If we can isolate a temperature-dependent lysogen that will grow only at 48° C it should be possible to make an unusual vehicle.

D. Site-specific endonucleases

Recently two restriction modification systems have been observed between B. subtilis 168 and other bacilli. Trautner et al. have isolated an effective system that inhibits infection of the R strain of B. subtilis by bacteriophage SPP1 propagated on B. subtilis 168 (27). The site-specific nuclease recognizes the sequence GGCC. Young, Radnay, and Wilson observed
CCGG
a restriction modification system between B. amyloliquefaciens and B. subtilis 168 (28). The endonuclease from B. amyloliquefaciens (20) recognizes the sequence GGATCC (29). More recently, two additional enzymes have
CCTAGG
been isolated from B. globigii (30). The recognition sequence is not known.

E. Advantages and liabilities of the B. *subtilis* system

a. Advantages

1. B. subtilis is nonpathogenic. Asporogenic deletion mutants are available to preclude the problem of persistence through sporulation.

2. The circular chromosomal map is well defined. At least 196 loci have been positioned.

3. The organism is commercially important in the fermentation industry.

4. Large numbers of organisms can be disposed of readily with minimal environmental impact.

5. Unlike E. coli, it lacks endotoxin in the cell wall. Therefore the cells can be used as a single cell protein source.

6. The frequency of transformation is very high, facilitating the detection of rare events.

7. A unique bacteriophage, ϕ3T, exists that carries a gene that can be readily purified for "scaffolding" experiments.

b. Disadvantages

1. The knowledge of genetics and physiology of plasmids and viruses is primitive compared with E. coli.

2. High-frequency, specialized transduction is not available as a means of gene enrichment.

Based on its promise, it seems appropriate, and not chauvinistic, to urge development of this system.

Prepared by:

Dr. Frank Young
University of Rochester

REFERENCES

1. Spizizen, J. 1958. Transformation of biochemically deficient strains of Bacillus subtilis by deoxyribonucleate. Proc. Nat. Acad. Sci. U.S.A. 44:1072-1078.

2. Lepesant-Kejzlarova, J., J.-A. Lepesant, J. Walle, A. Billault, and R. Dedonder. 1975. Revision of the linkage map of Bacillus subtilis 168: indications for circularity of the chromosome. J. Bacteriol. 121:823-834.

3. Young, F.E. and G. A. Wilson. 1975. Chromosomal map of Bacillus subtilis p. 596-614. In P. Gerhardt, R. N. Costilow, and H. L. Sadoff (ed.), Spores VI. American Society for Microbiology, Washington, D.C.

4. Wake, R. G. 1974. Termination of Bacillus subtilis chromosome replication as visualized by autoradiography. J. Mol. Biol. 86:223-231.

5. Harford, N. 1975. Bidirectional chromosome replication in Bacillus subtilis. J. Bacteriol. 121:835-847.

6. Bettinger, G. E. and F. E. Young. 1975. Transformation of Bacillus subtilis: Transforming ability of deoxyribonucleic acid in lysates of L-forms or protoplasts. J. Bacteriol. 122:987-993.

7. Thorne, C. B. 1962. Transduction in Bacillus subtilis. J. Bacteriol. 83:106-111.

8. Takahashi, I. 1961. Genetic transduction in Bacillus subtilis. Biochem. Biophys. Res. Commun. 5:171-175.

9. Yasbin, R. E. and F. E. Young. 1974. Transduction in Bacillus subtilis by bacteriophage SPP1. J. Virol. 14:1343-1348.

10. Shapiro, J. A., D. H. Dean and H. O. Halvorson. 1974. Low-frequency specialized transduction with Bacillus subtilis bacteriophage ϕ105. Virology 62:393-403.

11. Marmur, J., E. Seaman, and J. Levine. 1963. Interspecific transformation in Bacillus. J. Bacteriol. 85:461-467.

12. Young, F. E. and G. A. Wilson. 1972. Genetics of Bacillus subtilis and other gram-positive sporulating bacilli, p. 77-106. In H. O. Halvorson, R. Hanson, and L. L. Campbell (ed.), Spores V. American Society for Microbiology, Washington, D. C.

13. Chilton, M-D., and B. J. McCarthy. 1969. Genetic and base sequence homologies in bacilli. Genetics 62:697-710.

14. Wilson, G. A. and F. E. Young. 1972. Intergenotic transformation of the Bacillus subtilis genospecies. J. Bacteriol. 111:705-716.

15. Yamaguchi, K., Y. Nagata, and B. Maruo. 1974. Genetic control of the rate of α amylase synthesis in Bacillus subtilis. J. Bacteriol. 119:410-415.

16. Lovett, P. S., and F. E. Young. 1969. Identification of Bacillus subtilis NRRL B-3275 as a strain of Bacillus pumilus. J. Bacteriol. 100:658-661.

17. Lovett, P. S., and M. G. Bramucci. 1975. Plasmid deoxyribonucleic acid in Bacillus subtilis and Bacillus pumilus. J. Bacteriol. 124:484-490.

18. Lovett, P. S. 1973. Plasmid in B. pumilus and the enhanced sporulation of plasmid negative variants. J. Bacteriol. 115:291-298.

19. Young, F. E., M. T. Williams, and G. A. Wilson. Genetics of Bacillus subtilis. In D. Schlessinger (ed.) Microbiology 1976, in press.

20. Wilson, G. A. and F. E. Young. 1975. Isolation of a sequence-specific endonuclease (Bam 1) from Bacillus amyloliquefaciens H. J. Mol. Biol. 97:123-125.

21. Brown, L. Recombination analysis with purified endonuclease fragments in the RNA polymerase region of Bacillus subtilis. In D. Schlessinger (ed.), Microbiology 1976, in press.

22. Harris-Warrick, R. M., Y. Elkana, S. D. Ehrlich, and J. Lederberg. 1975. Electrophoretic separation of Bacillus subtilis genes. Proc. Nat. Acad. Sci. U.S.A. 72:2207-2211.

23. Kloos, W. F., and M. S. Musselwhite. 1975. Distribution and persistence of Staphylococcus and Micrococcus species and other aerobic bacteria on human skin. Applied Micro. 30:381-395.

24. Brown, W. C., and F. E. Young. 1970. Dynamic interactions between cell wall polymers, extracellular proteases and autolytic enzymes. Biochem. Biophys. Res. Commun. 38:564-568.

25. Clark, V. L. and F. E. Young. 1974. Active transport of D-alanine and related amino acids by whole cells of Bacillus subtilis. J. Bacteriol. 120:1085-1092.

26. Clark, V. L. and F. E. Young. Active transport in cells of B. subtilis 168: Loss of endogenously energized transport in auxotrophs deprived of D-alanine or glycerol. Submitted to J. Bacteriol.

27. Trautner, T. A., B. Pawlek, S. Bron, and C. Anagnostopoulos. 1974. Restriction and modification in B. subtilis: biologic aspects. Mol. Gen. Genet. 131:181-191.

28. Young, F. E., E. Radnay, and G. A. Wilson. Manuscript in preparation.

29. Wilson, G. A. and F. E. Young. Unpublished data.

30. Wilson, G. A., R. Roberts, and F. E. Young. Unpublished data.

31. Wilson, G. A. and F. E. Young. Restriction and modification in bacilli. In D. Schlessinger (ed.), Microbiology 1976, in press.

Polyoma and SV40 Virus

Polyoma virus is a virus of mice, and infection of wild mouse populations is a common event, for the virus has often been isolated from a high proportion of healthy adult animals, both wild and laboratory bred, of many colonies (Gross, L., Proc. Soc. Exp. Biol. 88, 362-368, 1955; Rowe, W. P., Bact. Rev. 25, 18-31, 1961). As far as is known the virus almost never causes a disease in these animals. However, when large quantities of the virus are inoculated into newborn or suckling mice or hamsters, a variety of solid tumors is induced (Gross, L., Oncogenic Viruses, Second Edition, Pergamon Press, NY).

Polyoma virus grows lytically in mouse cells in tissue culture. Thus mouse cells in culture are probably transformed only by virus particles that contain certain kinds of defective genomes. Cells of other rodent species, however, can be transformed by polyoma virus particles that contain complete genomes (Folk, W., J. Virol., 11, 424-431, 1973). The virus does not replicate to a significant extent in human cells in tissue culture (Eddy, B.E., Virol. Monogr., 7, 1-114, 1969; Pollack, R. E., Salas, J., Wang, R., Kusano, T., and Green, H., J. Cell Physiol. 77, 117-120, 1971). The resistance of the cells seems to be a consequence of the failure of the virus to absorb or uncoat. However even when naked viral DNA is introduced into the cells only an abortive cycle of replication ensues; early viral proteins are made, there is induction of cellular DNA synthesis, but no expression of late viral proteins is detectable (Gruen, R., Grassmann, M. and Grassmann, A., Virology, 58, 290-293, 1974).

There is no evidence that polyoma virus can infect humans (Hartley, J., Huebner, R., Parker, J. and Rowe, W. P., unpublished data). Thus no antibodies to the virus have been detected in people living in buildings that are infested with virus-infected mice, nor in laboratory workers who have been exposed to the virus for a number of years.

At most, a small segment of polyoma virus DNA shows weak homology with a portion of the late region of SV40 DNA (Ferguson, J. and Davis, R. W., J. Mol. Biol., 94, 135-150, 1975). However, there appears to be no genetic interaction between the two viruses and there is no immunological cross-reaction between the gene products of the two viruses.

SV40 causes persistent but apparently harmless infections of the kidneys of virtually all adult rhesus monkeys (Hsiung, G. D., Bact. Revs. 32, 185-205, 1968), it causes tumors when injected into newborn hamsters (Girardi, A. J., Sweet, B. H., Slotnick, V. B. and Hillemann, M. R., Proc. Soc. Exp. Biol. Med., 105, 420-427, 1964) and transforms cells of several mammalian species (including human). SV40 is able to infect humans since antibodies to the virus are found in a small proportion of the human population (Shah, K. V., Goverdhan, M. K. and Ozer, H. L., Am. J. Epid. 93, 291-298, 1970) and serum conversions have been noted in many laboratory personnel who have been exposed to the virus (Horvath, L. B., Acta Microbiol. Acta Sci. Hung. 12, 201-206, 1965).

Isolations of SV40 have been reported from humans, twice from patients suffering from the rare demyelinating disease, progressive multifocal leukoencephalopathy (Weiner, L., Herndon, R., Narayon, O., Johnson, R. T., Shah, K., Rubinstein, L. G., Prezozisi, T. J. and Conley, F. K., New England

J. Med. 286, 385-390, 1972) and apparently from a tumor of a person with metastatic melanoma (Soriano, F., Shelburne, C. E. and Gokcen, M., Nature, 249, 421-424, 1974). In other studies a non-structural antigen characteristic of papovaviruses, T antigen, has been detected in the nuclei of cells cultured from 2 meningiomas, while another SV40-specific antigen, U antigen, has been found in the cells of a third tumor of the same type (Weiss, A. F., Portman, R., Fisher, H., Simon, J. and Zang, K. D., Proc. Nat. Acad. Sci. USA 72, 609-613, 1975). Furthermore new papovaviruses have been isolated from the brains of patients with PML (JC virus - Padgett, B. L., Walker, D. L., zuRhein, G. M., Eckroade, R. I. and Dessel, B. H., Lancet 1, 1257-1260, 1971), from the urine of a patient carrying a renal allograft (BK virus - Gardner, S. D., Field, A. M., Coleman, D. V. and Hulme, B. Lancet 1, 1253-1257, 1971) and from a reticulum cell sarcoma and the urine of patients with the sex-linked recessive disorder, Wiskott-Aldrich syndrome (Takemoto, K. K., Rabson, A. S., Mullarkey, M. F., Blaese, R. M. Garon, C. F. and Nelson, D. J., Nat. Cancer Inst., 53, 1205-1207, 1974). All of these viruses which are distributed widely throughout human populations share antigenic and biological properties with SV 40; the virus particles are identical in size and architecture (Madeley, C. R., In Virus Morphology, Churchill-Livingstone, London, 134-135, 1972); the non-structural intracellular T antigen, which appears to be coded by the A gene of SV40 cross reacts extensively with antigens found in cells infected or transformed by BK or JC viruses; both JC and BK viruses induce tumors in newborn hamsters (Walter, D. L., Padgett, B. L., zuRhein, B. M., Albert, A. E. and Marsh, R. F., Science 181. 674-676,

1973; Shah, K. V., Daniel, R. W. and Strandberg, J., J. Nat. Cancer Inst. 54, 945-950, 1975); BK virus causes transformation of hamster cells in culture (Major, E.D., and DiMayorca, G., Proc. Nat. Acad. Sci. US, 70, 3210-3212, 1973; Portolani, M., Barbanti, A., Brodano, G. and LaPlaca, M.J., Virol. 15, 420-422, 1975) and is able to complement the growth of certain temperature-sensitive mutants of SV40 (Masion, B. H. and Takemoto, K. K., submitted for publication).

Further Work

At present, a potential eukaryotic vector of choice is polyoma virus. And while available information indicates that it fulfills all the necessary criteria, we recommend that the following subjects be further investigated:

1. The molecular mechanism of resistance of human cells to the virus
2. The extent of homology between polyoma virus DNA and the DNAs of human papovaviruses
3. The ability of human papovaviruses to complement defective polyoma virus genomes.

Report of a Working Group Consisting of:

Dr. Bernard Fields
Harvard University School of Medicine

Dr. Thomas J. Kelly, Jr.
Johns Hopkins University
School of Medicine

Dr. Andrew Lewis
National Institute of Allergy
and Infectious Diseases

Dr. Malcolm Martin
National Institute of Allergy
and Infectious Diseases

Dr. Robert Martin
National Institute of Arthritis,
Metabolism, and Digestive Diseases

Dr. Elmer Pfefferkorn
Dartmouth Medical School

Dr. Wallace P. Rowe
National Institute of Allergy
and Infectious Diseases

Dr. Aaron Shatkin
Roche Institute of Molecular
Biology

Dr. Maxine Singer
National Cancer Institute

Summary of the Workshop on the DESIGN AND TESTING OF SAFER PROKARYOTIC VEHICLES AND BACTERIAL HOSTS FOR RESEARCH ON RECOMBINANT DNA MOLECULES

Torrey Pines Inn, La Jolla, California

The development of techniques for the cloning of DNA from both prokaryotic and eukaryotic organisms in bacteria has had great impact on research in biology and medicine and promises extraordinary social benefits. The biohazards involved in the use of this technology in many instances are very difficult to assess. For this reason codes of practice are being formulated in the United States and other countries for the conduct of those experiments that present a potential biohazard. One of the requirements for conducting certain cloning experiments is the use of safer vector (bacteriophage or plasmid) -host systems, i.e., vector-bacterium systems that have restricted capacity to survive outside of controlled conditions in the laboratory. Approximately sixty scientists from the United States and several foreign countries participated in a workshop on the Design and Testing of Safer Prokaryotic Vehicles and Bacterial Hosts for Research on Recombinant DNA Molecules at La Jolla, California, on 1 to 3 December, 1975. The workshop was sponsored by the Research Resources Branch of the National Institute of Allergy and Infectious Diseases. The purposes of the meeting were the exchange of recent data on the development of safer prokaryotic host-vector systems, devising methods of testing the level of containment provided by these systems and exploring the various directions that future research should take in the construction of safer bacterial systems for the cloning of foreign DNA.

The first session of the workshop, chaired by W. Szybalski (University of Wisconsin), was devoted to bacteriophage vectors. Szybalski outlined the main safety features of the two-component, phage-bacterial system, in which the host bacteria offer the safety feature of not carrying the cloned DNA, and the phage vectors cannot be propagated in the absence of an appropriate host. There are two primary escape routes for the clones of foreign DNA carried by the phage vector: (1) establishment of a stable prophage or plasmid in the laboratory host used for phage propagation, and subsequent escape of this self replicating lysogen or carrier system, and (2) escape of the phage vector which carries the cloned DNA and its subsequent productive encounter with a suitable host in the natural environment. The general consensus was that to ensure safety, both routes should be blocked by appropriate genetic modifications. For phage λ, route (1) can be blocked by phage mutations that interfere with lysogenization (*att*$^-$, *int*$^-$, *c*I$^-$, *c*III$^-$, *vir*) and plasmid formation (N^+, *nin*R, *v*S, *ri*^{c}, *c*I7, *O*ts, *cro*ts), and by mutations on the *Escherichia coli* host that affect these processes (*att*B$^-$, *dnc*Ats) and host survival. Route (2), [which is of low probability since λ phages do not survive well in natural environments (no λcI phage was recovered after ingestion of 10^8-10^{11} particles), are killed by desiccation, and have a low chance to encounter a naturally sensitive host] can be blocked further by the following phage modifications: (a) mutations which result in extreme instability of the infectious phage particles under all conditions other than those specially designed for phage propagation in the

laboratory (e.g., high concentrations of putrescine or some other compound), or (b) employing phage vectors in which the tail genes are deleted and which permit propagation of only the DNA-packed heads; only under laboratory conditions could such heads be made transiently infectious by rejoining them with separately prepared tails. The high instability of the phage would minimize the possibility of transfer of the cloned genes into receptive bacteria found in nature. Moreover, the propagation of the phage can be blocked by many conditional mutations, which would be designed to block any secondary route of escape, mainly depending on transfer of the cloned DNA into another phage or bacterial host. It was recommended further that the vector be designed in such a manner as to permit easy insertion and monitoring of the foreign DNA and rapid assay of the safety features and give a high yield of cloned DNA (not less than 10^{11} molecules per ml). There also was general agreement that host-phage systems other than *E. coli* should be considered, especially those restricted to very rare and unusual environments. Also, plasmids derived from phage vectors and which give very high DNA yields while exhibiting safety features, e.g., λdv*cro*ts, should be considered as vehicles for cloned DNA.

Szybalski and S. Brenner (Cambridge University) stressed that research on recombinant DNA molecules may lend itself to very simple and inexpensive mechanical containment, e.g., a small sealed glove box, since all the vectors that carry such recombinant molecules possibly can be both created and destroyed in such a box, while development of special methods might permit study of many properties of the recombinant

DNA, without ever removing it from the box.

These safety features were reflected in the subsequent presentations. F. Blattner and W. Williams (University of Wisconsin) described four specially constructed λ-φ80 phages which incorporate many of these safety features, and which they named Charon phages, for the mythical boatman of the river Styx. Some of these highly contained phages give yields of over 10^{11} particles/ml. R. Davis, J. Cameron and K. Struhl (Stanford University) found that λ phages that carry foreign DNA never grow as well as the parental vector, which would select against their survival in nature. They also reported that some eukaryotic genes could be expressed in *E. coli*, partially compensating for deficiencies in the histidine pathway or in *polA* or *lig* functions. These investigators surveyed over 1000 strains of *E. coli* isolated in the natural environment and did not find a single strain that could support propagation of the *λvir* vector.

V. Bode (Kansas State University) discussed the possibility of growing tail-free λ heads. Such heads, which are packed with DNA, are very fragile, unless stored in 0.01 M putrescine buffer. Head yields close to 10^{11}/ml could easily be attained and, when required, heads could be quantitatively rejoined with separately supplied tails under special laboratory conditions. W. Arber, D. Scandella and J. Elliott (University of Basel) described bacterial host mutants that permit efficient infection only by phages with a full complement of DNA. This permits selecting for vectors that carry long fragments of foreign DNA.

K. Matsubara, T. Mukai and Y. Takagi (University of Osaka and Kyushu University), and G. Hobom and P. Phillippsen (University of Freiburg and Stanford University) described various defective λ plasmids (λdv) that could be used as efficient vectors. Matsubara has shown that temperature-sensitive *cro* mutations permit obtaining between 1000 and 3000 cloned molecules per cell and at the same time result in killing of the carrier cells at body temperature. The mutations *O*ts and *P*ts were also evaluated as safety features. Phillippsen described many new λdv plasmids created by cutting λ DNA with *Hind*III and *Bam*I restriction endonucleases followed by ligation. The final talk by F. Young, G. Wilson and M. Williams (University of Rochester) summarized the progress on the development of safer *Bacillus subtilis* host mutants and phages, especially ϕ3, as vectors. New restriction nucleases, Bgl-1 and Bgl-2, were also described.

The morning session on bacteriophage vectors was followed by a session on plasmid vectors that was chaired by D. Helinski (University of California, San Diego). Helinski presented the following properties as highly desirable characteristics of a safer plasmid vehicle: (a) non-conjugative; (b) non-mobilizable or poorly mobilizable by a conjugative plasmid; (c) possesses little or no extraneous genetic information; (d) poorly recombines or does not recombine with the chromosome of the host cell; (e) provides no selective advantage to the host cell or the selective property is conditional; and (f) possesses mutations that restrict its maintenance to a specific host, prevent replication at mammalian body temperature and/or provide the plasmid with the

capability of killing any cell to which it might be transmitted other than the host cell. V. Hershfield (University of California, San Diego) described the properties of a variety of derivatives of the ColE1 plasmid and the broad-host range, P-type plasmid, RK2. One of the ColE1 derivatives, ColE1-*trp*, constructed in collaboration with C. Yanofsky and N. Franklin (Stanford University) provides the means to use the tryptophan genes of *E. coli* as a selective marker in transformation with recombinant DNA in situations where it is desirable to avoid antibiotic resistance genes. In addition, Hershfield described collaborative work with H. Boyer that resulted in the development of a mini-ColE1 plasmid and derivatives of this plasmid (mini-ColE1-*kan* and mini-ColE1-*trp*) as cloning vehicles. Finally, she described the temperature-sensitivity properties of *trp* and *kan* derivatives of a temperature-sensitive replication mutant of ColE1 isolated by J. Collins (Molecular Biology Institute, Stockheim) and hybrid ColE1 plasmids carrying the *Eco*RI generated Cts fragment of bacteriophage λ-trp61.

J. Carbon (University of California, Santa Barbara) described a replica plating method that greatly facilitates the detection of *E. coli* clones bearing ColE1 plasmids. The procedure, which utilizes the F_1 plasmid to promote the transfer of a hybrid ColE1 plasmid to a suitable auxotrophic recipient, was successful in identifying clones bearing hybrid plasmids carrying a number of different regions of the *E. coli* chromosome. The contributions of A. J. Clark and collaborators (University of California, Berkeley) were relevant to the problem of the mobilization and subsequent transfer of non-conjugative plasmids

carrying foreign DNA of a potentially hazardous nature. Clark described the variations in transmission frequencies between the non-conjugative plasmids pSC101, pML31, pSC138 and a number of pSC101 hybrids containing various *Eco*RI fragments of F when the conjugal transfer of these plasmids was promoted by several different conjugative plasmids.

I. C. Gunsalus and collaborators (University of Illinois) and A. Chakrabarty (General Electric Research and Development Center) described the properties of a variety of plasmids isolated from *Pseudomonas putida*. These contributions were followed by a discussion on the merits of developing plasmid-host systems involving *Pseudomonas* strains that naturally exhibit unusual growth requirements. Similar studies with plasmids isolated from *Bacillus megaterium* by B. Carlton (University of Georgia) from *B. subtilis* by P. Lovett (University of Maryland) and other naturally occurring *Bacillus* species by W. Goebel and K. Bernhard (Microbiology Institute, Wurzburg) were discussed and their further development as plasmid-host cloning systems was explored. It was clear from these presentations that considerable progress has been made recently in the identification and characterization of a variety of plasmid elements that occur naturally in *Pseudomonas* and *Bacillus* species. Several of the plasmids described show considerable promise as plasmid cloning systems involving a host other than *E. coli*.

A third session on the ecology and epidemiology of vector-host systems was chaired by S. Falkow (University of Washington). This workshop emerged, in part, from expressed fears that microorganisms

containing cloned fragments of foreign DNA may potentially pose a threat to health or disrupt the normal ecological chain in some manner. Consequently, this session was devoted to a review of currently available information on the ecology and epidemiology of *E. coli* and related bacterial species since it was recognized that *E. coli* K-12 would be the prokaryotic host most commonly employed in the cloning of DNA molecules in the immediate future. F. Ørskov (Escherichia Reference Center, Copenhagen) reviewed the state of *E. coli* serotyping and what has been learned about the distribution of *E. coli* types in health and disease. Only certain *E. coli* types are generally recognized as good colonizers of the human gut and such strains come from a handful of the 160 well defined O (lipopolysaccharide) antigen types and invariably possess K (acidic polysaccharide capsule) antigens. Some serotypes apparently have become disseminated worldwide and possibly represent the proliferation of a bacterial clone because of, as yet unknown, selective pressures. In contrast, *E. coli* K-12 has no detectable O or K antigens and is considered to be rough. This may account, at least in part, for its demonstrable poor ability to colonize the human or animal gut. However, R. Freter (University of Michigan) pointed out that we still remain largely ignorant of the factors which control intestinal *E. coli* populations. Freter also noted that while adherence to the mucosal surface of the small intestine is important in the pathogenesis of *E. coli* diarrheal disease, the 'normal' long-lasting symbiotic relationship between a mammalian host and bacterium is established in the cecum and colon. It is in these locations that factors come into play to determine

whether an *E. coli* strain passing through the intestine will become successfully implanted or whether it will be quickly eliminated in the feces. The factors controlling implantation include competition for substrates, inhibitors and the physiological state of the organism when it reaches the large bowel. For example, ingested *E. coli* previously grown under usual laboratory conditions fare poorly while cells of the same strain 'pre-adapted' in Eh, pH, etc., often colonize well. Freter has developed a continuous flow culture model which may be useful in studying the mechanisms of implantation. Falkow reviewed the pathogenicity of *E. coli*. *E. coli* causes diarrheal disease either by direct invasion of the bowel epithelium or by elaboration of enterotoxin(s). While invasive *E. coli* appear to owe their pathogenicity to a constellation of at least five unlinked chromosomal gene clusters, toxigenic *E. coli* species generally owe their pathogenicity to the possession of two species, Ent and K. The introduction of Ent and K plasmids may be sufficient to convert a normal wild-type *E. coli* into a strain now capable of causing overt clinical disease. However, the introduction of these plasmids into *E. coli* K-12 sublines had no discernible effect on their ability to cause disease, although the K-12 strains could now better colonize calves. Despite the observation that *E. coli* K-12 did not appear to offer a significant hazard as a potential enteric pathogen even when it possessed well-defined determinants of pathogenicity it was emphasized by Ørskov, Freter and Falkow that *E. coli* K-12 strains carrying recombinant DNA molecules could still act as effective genetic donors *in vivo* and still posed a significant problem

requiring control. E. Geldreich (U.S. Environmental Protection Agency, Cincinnati, Ohio) discussed the possible outcomes of the release of *E. coli* containing recombinant DNA molecules into the aquatic environment and concluded that total reliance cannot be placed on sewage treatment and the natural self-purification capacity of receiving waters to limit potential hazards. While these are realistic barriers to the dissemination of *E. coli* and associated fecal organisms via the water route, they are not infallible because of technological limitations, improper operational practices and system overloading. Finally, M. Starr (University of California, Davis) described the numerous genera of gram-negative bacteria found naturally occurring in the soil and on plants. He stated that most of these organisms do not appear to be a reasonable alternative to *E. coli* K-12 as a host for recombinant DNA molecules. Indeed, Starr pointed out that since such genera as *Erwinia, Rhizobium* and *Agrobacterium* are known to conjugate with *E. coli*, the potential dissemination of recombinant DNA molecule includes a greater spectrum of microorganisms than just enteric species.

The fourth session of the workshop, chaired by R. Curtiss III (University of Alabama), was concerned with the construction of safer bacterial hosts for DNA cloning. The goals in constructing safer host strains enumerated at the beginning of the session included introduction of mutations that would: (a) preclude colonization in normal ecological niches; (b) preclude cell wall biosynthesis except in specially defined media; (c) cause degradation of genetic information in normal ecological niches; (d) cause vectors to be host-dependent; (e) minimize transmission of recombinant DNA to other strains in normal ecological niches;

(f) increase usefulness for recombinant DNA molecule research; and

(g) permit monitoring.

Most of the progress in developing safer hosts has been achieved with *E. coli* K-12, although F. Young described a *B. subtilis* strain with a deletion for sporulation genes which readily undergoes autolysis. The strain also has defects in genes for purine and TTP biosynthesis and a mutation conferring a D-alanine requirement can be introduced to cause cell wall biosynthesis to be defective. This strain may be defective in transformation, however, and therefore might be useful only with a phage vector which has yet to be developed and/or discovered.

A. I. Bukhari (Cold Spring Harbor Laboratory) described the use of the *dapD8* mutation in *E. coli* K-12 to block cell wall biosynthesis and another non-reverting mutation which causes sensitivity to bile salts and detergents. The *dapD8* allele is the most stable *dap* point mutation known, although it does revert at frequencies of 10^{-8} to 10^{-9}. The mutation conferring bile salts sensitivity was obtained after Mu-1 infection of an Hfr strain and, although exhibiting the theoretically useful properties of ease of DNA isolation and inability to survive in the intestinal tract, might be due to Mu insertion which would compromise its use for safe strain construction.

Curtiss reported on the work performed by him and his coworkers in constructing and testing numerous strains with different mutations. Survival of strains *in vivo* was tested by feeding rats 10^{10} cells in milk by stomach tube. Δ*pur* mutations did not reduce strain titers in feces whereas Δ*thyA*; Δ*thyA drm*; and Δ*thyA dra* mutations gave 10^{2}-fold, 10^{2}-fold and 10^{5}-fold reductions, respectively, in strain titers in

feces. Strains with Δ*thyA* mutations also exhibited thymineless death in *in vitro* tests. Since strains with the *dapD8* allele can revert to Dap$^+$, strains were constructed with both the *dapD8* and Δ*bioH-asd* mutations. These strains have not been observed to revert to Dap$^+$ but can survive passage through the rat intestine and in growth media lacking diaminopimelic acid but containing NaCl and 0.5% usable carbon sources. This survival was due to the production of the mucopolysaccharide, colanic acid, which permits many of the cells to grow and survive as spheroplasts. A Δ*gal-chl*^{r} mutation (also deletes λ*att*, *bio* and *uvrB* genes) was introduced which blocks colanic acid biosynthesis and leads to no detectable survivors in media lacking diaminopimelic acid or following passage through the rat intestine. The *dapD8* Δ*bioH-asd* Δ*gal-chl*^{r} strains are more readily lysed, transform at higher frequencies and are conjugation-defective in matings with donors possessing conjugative plasmids in the P, W and O incompatibility groups but Con$^+$ as recipients for F, I and T group plasmids when compared to the *dap*$^+$ *gal*$^+$ parent strain. Strains with *endA* mutations were also observed to exhibit increased transformation frequencies. Attempts to introduce temperature-sensitive *polA* alleles into strains to block replication of ColE1 cloning vectors at elevated temperatures and to cause DNA degradation at elevated temperatures in the presence of *recA* and Δ*thyA* alleles often do not have the same properties in the constructed strains as in the strains in which the allele was originally induced. Many mutations causing a Con$^-$ phenotype have been investigated, but many of these revert and/or do not exhibit a Con$^-$ phenotype in matings with donors possessing

conjugative plasmids of the incompatibility groups commonly found in enteric microorganisms. Some Con$^-$ mutants exhibit increased sensitivity to bile salts; thus, the mutant described by Bukhari may also exhibit a Con$^-$ phenotype. All of the strains constructed by the Curtiss group are SuII$^+$ and most have mutations abolishing restriction alone or both restriction and modification. Thus, sufficient information is now known to construct a usable safer *E. coli* K-12 host. Curtiss and collaborators are now introducing the Δ*thyA* and *dna* mutations into their *dapD8 ΔbioH-asd Δgal-chl*^{r}*-uvrB hsr nalA*^{r} (for ease in monitoring) Su$^+$ λ^r φ80^r strain to accomplish this objective.

The final session involved a general discussion of some of the major points raised previously in the workshop. There was general agreement at this session that both plasmid-host and phage-host systems have been developed that should meet the criteria of an EK2 system specified by the National Institutes of Health guidelines for research on recombinant DNA molecules. Additional testing is required to confirm the EK2 properties of these available systems, but it is anticipated that these vector-host systems will meet these tests.

Dr. Donald R. Helinski
University of California, San Diego

Dr. Roy Curtiss III
University of Alabama

Dr. Stanley Falkow
University of Washington

Dr. Waclaw Szybalski
University of Wisconsin